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EDITED BY
SIMON FLEXNER, M.D.

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A USEFUL HEART METHOD.

By CECIL K. DRINKER, M.D.

(*From the Laboratory of Applied Physiology of Harvard Medical School, Boston.*)

PLATES 83 AND 84.

(Received for publication, February 21, 1921.)

In order to accomplish certain experiments upon the relation of the pulmonary circulation to the air space in the lungs, it became necessary to devise a method in which the heart was exposed and the breathing normal. The procedure described below fulfills these requirements.

Under urethane anesthesia (2 gm. per kilo) and artificial respiration, an elliptical section is cut from the anterior aspect of the thorax of a cat. This piece must be taken out neatly and should leave the uppermost and lowest rib attachments to the sternum and sternal cartilage intact. The heart is then seen beating within the pericardium and the removed section of chest wall should be but slightly larger than the heart itself, so that the organ is framed by the margin of the opening. The anterior aspect of the pericardium is now slit exactly in the midline, and the edges of the pericardial opening attached to the margin of the thoracic window by three strong sutures on either side and one at each extremity. Then, by use of a long continuous suture, the pericardium is sewed to the margins of the window and is thus made to close the opening completely. It will be found to do more than this; that is, there will be a considerable amount of loose pericardial membrane below the heart, a desirable situation since the suturing should not involve serious tension at any point.

The chest wall, which has been closed in this manner anteriorly by the fibrous pericardial membrane, is now stretched laterally by traction on the stay sutures first placed, and superiorly and inferiorly by the stay sutures at the ends of the incision. The retraction so accomplished throws the heart forward where it may be handled with

greater readiness, and takes up the pericardial slack so that a reasonably rigid anterior chest wall is provided. Fortunately, the vessels entering the heart do so posteriorly, and lifting the heart forward in the manner described does not seem to affect its filling. Before discontinuing artificial respiration it is advantageous to withdraw air from the thorax with a syringe in order to assist in the reestablishment of negative intrathoracic pressure.

Fig. 1 shows the preparation as it appears when finished. Obviously one may readily adjust a cardiometer in such a heart technique and manipulate the cardiac vessels, etc., in connection with procedures in which it may be desirable to measure normal respiration at the same time.

Fig. 2 is a record made during the course of an experiment in which a urethanized cat, with heart exposed in the manner described, remained in good physiological condition from 10.35 a.m. to 3.20 p.m., and was then killed with chloroform. The lungs were not edematous at death and the animal would apparently have done well through many more hours.

In this tracing it is noticeable that the blood pressure never reached so high a level after as before operation, and this has invariably been our experience in using the method described. The height attained, however, has always been as great as under artificial respiration, and frequently has exceeded that level. The technique has been employed in the cat alone, but its ease of accomplishment in this animal leaves little doubt of its usefulness in other laboratory animals.

EXPLANATION OF PLATES.

PLATE 83.

FIG. 1. Method of exposing the heart under normal breathing.

PLATE 84.

FIG. 2. Upper tracing, respiration; inspiration written downward; middle tracing, arterial blood pressure; lower tracing, signal magnet ½ minute intervals. Tracing I, normal blood pressure. Tracing II, transition from artificial respiration to normal respiration. Tracing III, condition at 11 a.m. Tracing IV, condition at 12.35 p.m.

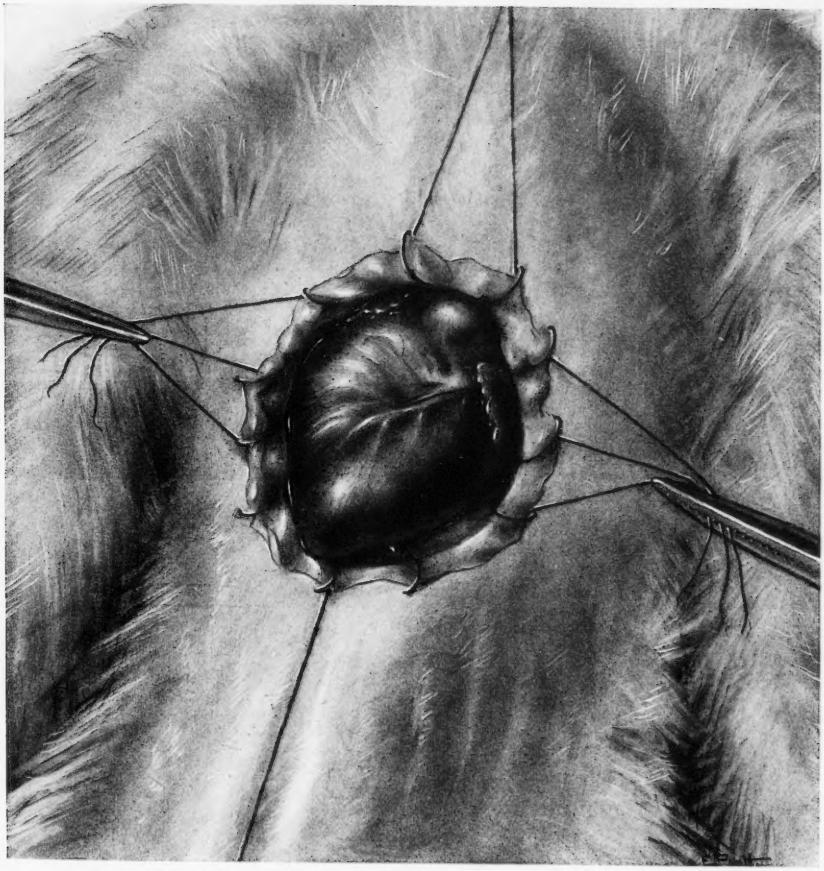

FIG. 1.

(Drinker: Useful heart method.)

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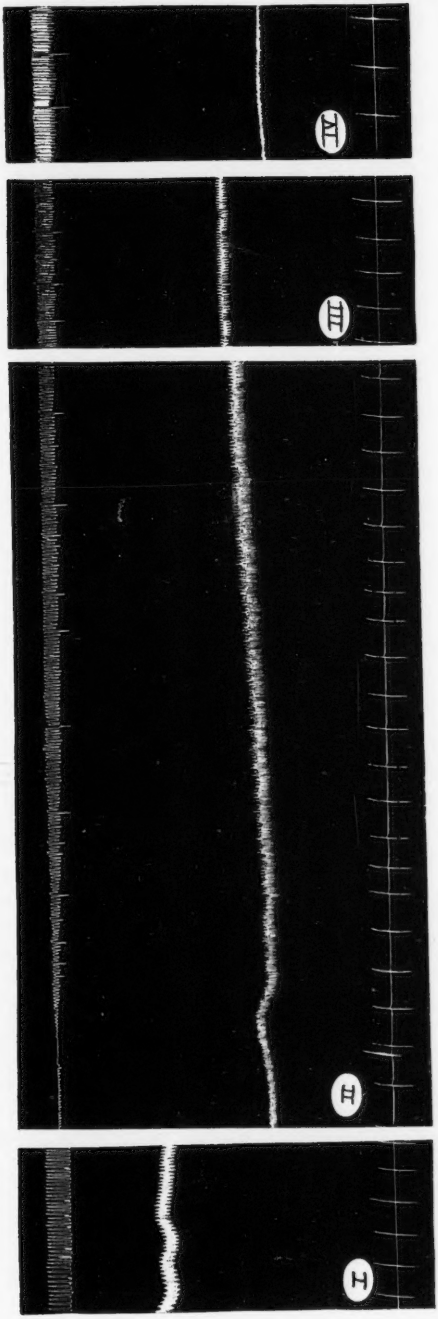

FIG. 2.

(Drinker: Useful heart method.)

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AN IMPROVED ANAEROBE JAR.

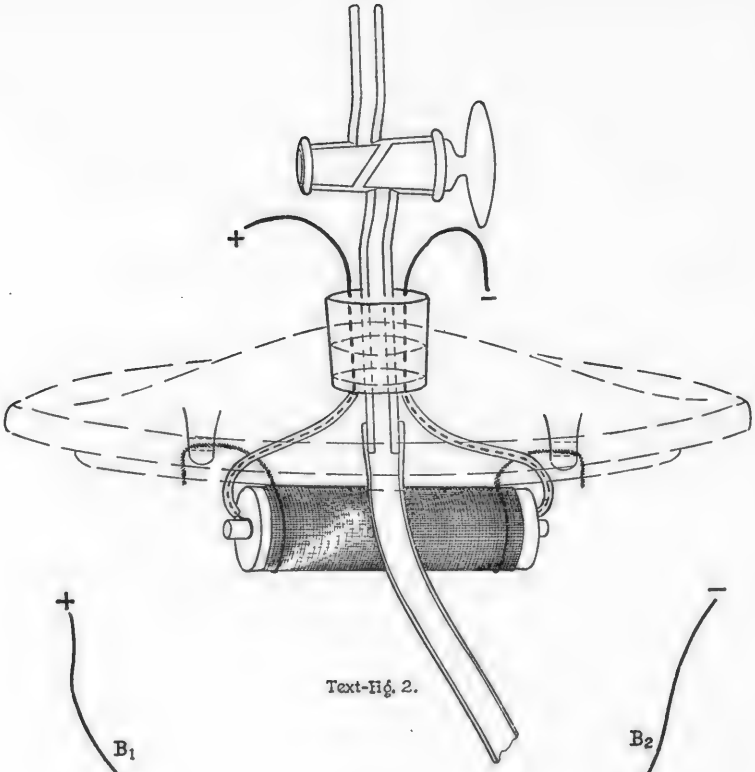
By J. HOWARD BROWN, Ph.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

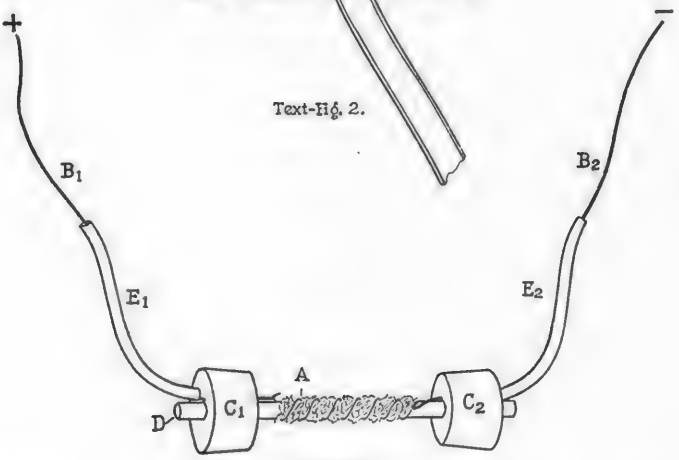
(Received for publication, February 23, 1921.)

Laidlaw (1) introduced the use of platinized carbon as a catalyzer for the combustion of oxygen and hydrogen to produce conditions favorable to the growth of anaerobic bacteria. His method was adapted for use with single tube cultures only. McIntosh and Fildes (2, 3) utilized the principle for the growth of anaerobes in jars. Their catalyzer consists of a small amount of platinized or palladinized asbestos wrapped in a piece of wire gauze and suspended from the lid of the jar. Hydrogen is introduced into the jar through a stop-cock in the lid. The method of McIntosh and Fildes was modified by W. G. Smillie (4) who enclosed the platinized asbestos in a small perforated glass bulb attached to the end of the tube through which the hydrogen is introduced into the jar, the hydrogen thus entering the jar through the asbestos. Fildes (5) has published a very useful review of these and other methods for the growth of anaerobic bacteria.

In the above mentioned methods the catalyzer must be heated in a flame, the tube or jar quickly closed, and the hydrogen introduced before the catalyzer has cooled, or else combustion does not occur. When the reaction has once started, however, the heat of combustion is sufficient to carry it to completion. There may, however, remain in the recesses of the jar or tubes or within the media traces of oxygen which diffuse out into the jar very slowly after the catalyzer has cooled and combustion has ceased. It is to be noted that Smillie's jar provided for intimate contact of the hydrogen but not of the oxygen with the catalyzer. He may have experienced some difficulty in this regard for in describing another form of apparatus he says: "The following method was devised to remove all the oxygen." The platinized asbestos was wrapped in a coil of fine nichrome wire the ends of which were joined to larger wires which passed upward through a rubber stopper. Hydrogen was introduced into a tube of inoculated solid medium and the rubber stopper inserted. The catalyzer was heated by passing an electric current through the coil surrounding it. Smillie points out that the tube may be set aside for a while and the asbestos reheated to ignite any residual oxygen. Apparently he got the most perfect anaerobic conditions with this apparatus. He did not utilize an electrically heated coil in jars in which many cultures could be enclosed, probably because of the danger of explosion when larger volumes of hydrogen and oxygen are used.



Text-Fig. 2.



Text-Fig. 1.

TEXT-FIG. 1. Showing the construction of the catalyzer coil. *A*, the fine nichrome wire coiled about the platinized or palladinized asbestos. B_1 and B_2, larger copper wires joined to the ends of the nichrome wire. C_1 and C_2, rubber stoppers. *D*, the core of glass tubing. E_1 and E_2, small rubber tubing serving as insulation.

TEXT-FIG. 2. Showing the coil enclosed by fine copper wire gauze and in position beneath the lid of the anaerobe jar.

In the apparatus to be described the danger of explosion is eliminated by completely enclosing the asbestos and coil of nichrome wire in a copper wire screen which does not come in contact with the coil or asbestos at any point. This introduces the principle of the Davy safety lamp. The apparatus is illustrated in Text-figs. 1 and 2, and is made as follows:

A piece of fine nichrome wire (B. and S. gauge No. 28) (Text-fig. 1, *A*) is joined at each end with pieces of larger copper wire (B_1 and B_2). One of the copper wires (B_1) is inserted through a No. 1 one-hole rubber stopper (C_1) and beside the wire is inserted also the end of a short piece of small glass tubing (*D*) holding the wire in the position shown in Text-fig. 1. Some palladinized asbestos is spread out onto a square of lens paper. This is then wrapped about the center of the glass tube and held in place by coiling the nichrome wire around it. The other copper wire (B_2) is then passed through another rubber stopper (C_2) which is placed over the other end of the glass tube. Pieces of small rubber tubing provide insulation for the copper wires at E_1 and E_2. A piece of fine copper wire gauze is rolled around the entire core and held in place by wires twisted about the stoppers at each end of the gauze. The twisted ends of these wires serve to fasten the cell to the lid of the jar as shown in Text-fig. 2. An ordinary round museum specimen jar is used. A one-hole rubber stopper carrying a glass stop-cock is inserted into a hole bored in the lid of the jar. The two copper wires coming from the coil are run up through the rubber stopper on either side of the stop-cock. This is easily done by sticking a large hypodermic needle down through the stopper, running the copper wire up through the bore of the needle and then withdrawing the needle, leaving the wire in place. In time leaks are likely to appear around the stopper, the wires, or the stop-cock, so that cement or sealing wax should be placed over and around the rubber stopper. From the lower end of the stop-cock a rubber tube leads to the bottom of the jar to insure a good mixture of the oxygen with the hydrogen entering the jar.

In use, the lid is clamped down onto the jar of cultures over a gasket of "plasticine" modeling clay. An electric current is connected with the two protruding copper wires and hydrogen is run into the jar through the stop-cock under pressure of about 5 pounds.

Combustion is soon manifested by the collection of moisture inside the jar and by the lid becoming quite warm. This is allowed to continue until the flow of hydrogen ceases, as may be detected by observing no more bubbles in the wash bottles of the hydrogen apparatus. 20 or 30 minutes are usually sufficient, after which the stop-cock is closed, the electric current disconnected, and the jar incubated. At any time during incubation the electric current may again be passed through the coil to consume any residual oxygen. Sufficient hydrogen for this purpose will remain in the jar. A tube of gelatin tinted with methylene blue and decolorized in boiling water just before being sealed within the jar serves as an indicator of the presence or absence of oxygen.

An electric light current of 110 volts reduced by passage through a 60 watt Mazda lamp has been used for heating the coil. Very little hydrogen is required since none is passed into and out of the jar as is the case with the Novy jar.

A number of coils and jars as described have been in use for several months. There have been no accidents due to explosion such as have been known to occur with other jars employing the combustion principle. Whenever perfect anaerobic conditions have not been attained the failure has been due to small leaks in the stopper or stop-cock. The coils have shown no deterioration with use or age. The bit of lens paper used to hold the asbestos in place when the nichrome wire is being coiled about it is burned off with the first passage of the electric current. Similar cells in which the hydrogen was introduced through the asbestos into the cell were made but found to possess no advantage over the one described.

SUMMARY.

There has been described a modification of the anaerobe jars of McIntosh and Fildes and of Smillie in which the oxygen is consumed by combustion with hydrogen under the catalytic action of platinized or palladinized asbestos.

The special advantages of the apparatus described reside in its greater safety and in the fact that the catalyzer is heated electrically after the jar is closed and may be reheated at any time during incubation without opening the jar.

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3. McIntosh, J., and Fildes, P., Nouvelle méthode d'isolement et de culture pour les microbes anaérobies, *Compt. rend. Soc. biol.*, 1916, lxxix, 293.
4. Smillie, W. G., New anaerobic methods, *J. Exp. Med.*, 1917, xxvi, 59.
5. Fildes, P., Methods for cultivating the anaerobic bacteria, *Med. Research Com., Nat. Health Insurance, Special Rep. Series, No. 12*, 1917, 59.

ETIOLOGY OF YELLOW FEVER.

XIII. Behavior of the Heart in the Experimental infection of Guinea Pigs and Monkeys with Leptospira icteroides and Leptospira icterohæmorrhagiæ.

By ALFRED E. COHN, M.D., and HIDEYO NOGUCHI, M.D.

(*From the Hospital and Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 85 and 86.

(Received for publication, March 9, 1921.)

It is well known that in patients suffering from yellow fever the rate of the heart has a tendency to be slow. This phenomenon was regarded by Faget,[1] who was among the first to call attention to it, and by Touatre[2] as pathognomonic in this disease, especially in its early stage. In the second stage, the rate falls still further. It may be as low as 30 or 40 per minute, and may continue slow during convalescence. In fatal cases in the final stage it may be either rapid or slow. In the curves of patients published by Noguchi[3] and by Elliott,[4] these relations are seen. Sarti[5] gives, however, high rates for ten of the eleven cases he reports. It is said that death is seldom due to heart failure. At autopsy, lesions of the heart are not constantly found, except perhaps the degenerative changes and frequent

[1] Faget, J. C., Monographie sur le type et la spécificité de la fièvre jaune établie avec l'aide de la montre et du thermomètre, Paris, 1875.

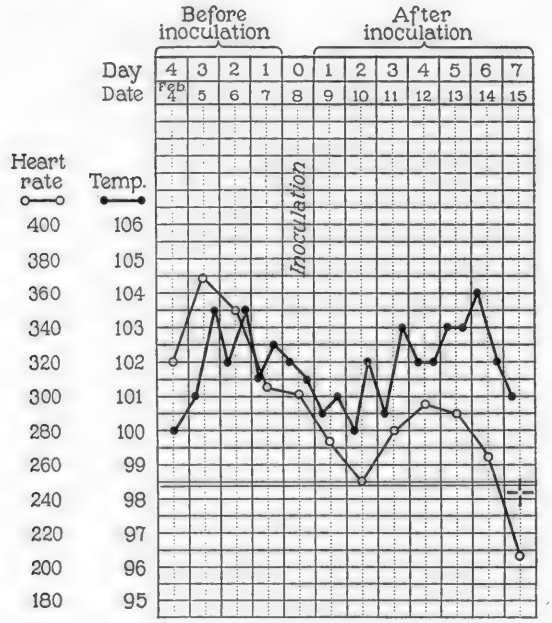
[2] Touatre, J., Yellow fever; clinical notes, translated by Chassaignac, C., New Orleans, 1898.

[3] Noguchi, H., Etiology of yellow fever. I. Symptomatology and pathological findings of the yellow fever prevalent in Guayaquil, *J. Exp. Med.*, 1919, xxix, 547.

[4] Elliott, C. A., A clinical study of yellow fever. Observations made in Guayaquil, Ecuador, in 1918, *Arch. Int. Med.*, 1920, xxv, 174.

[5] Sarti, J., Contribucion al estudio de la fiebre amarilla, Tesis, Guatemala, 1919.

endocardiac or pericardiac ecchymoses associated with fever. Recent studies on infectious jaundice[6] indicate, however, that in that disease also the pulse rate is usually slow in proportion to the temperature; one case is reported in which the pulse fell as low as 38. This holds true even in cases without jaundice.



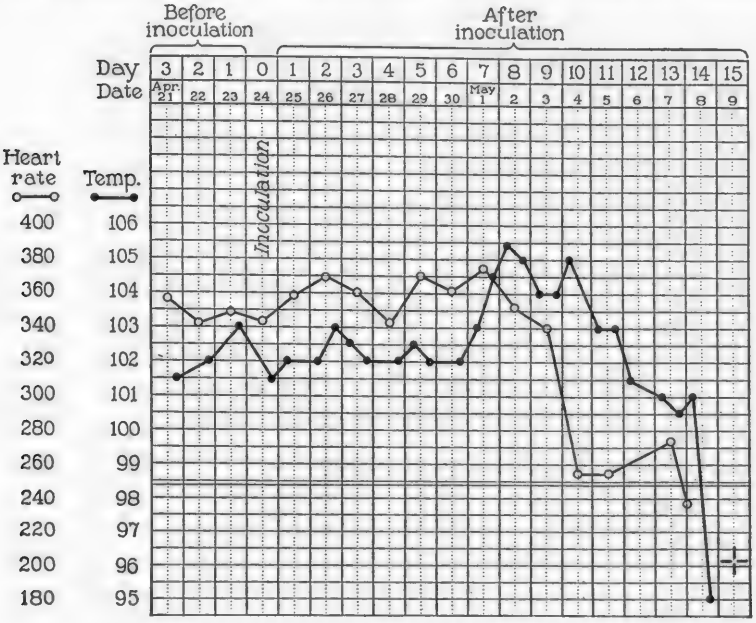
TEXT-FIG. 1. Temperature and heart rate curves of Guinea Pig 3, inoculated with *Leptospira icteroides*.

Since the successful transmission of yellow fever to animals, observations have been made of the rate and of the behavior of the heart in animals experimentally infected with *Leptospira icteroides* from yellow fever cases. Records were taken electrocardiographically.

[6] Stokes, A., Ryle, J. A., and Tytler, W. H., Weil's disease (spirochætosis ictero-hæmorrhagica) in the British Army in Flanders, *Lancet*, 1917, i, 142. Ryle, J. A., Spirochætosis icterohæmorrhagica (formerly known as Weil's disease). A clinical analysis of fifty-five cases, *Quart. J. Med.*, 1921, xiv, 139.

By this method the rate can be accurately counted and the mechanism of slowing, if it occurs, can be analyzed. For comparison with the animals inoculated with *Leptospira icteroides* (yellow fever) a certain number of others infected with *Leptospira icterohæmorrhagiæ* (infectious jaundice) were included in the study.

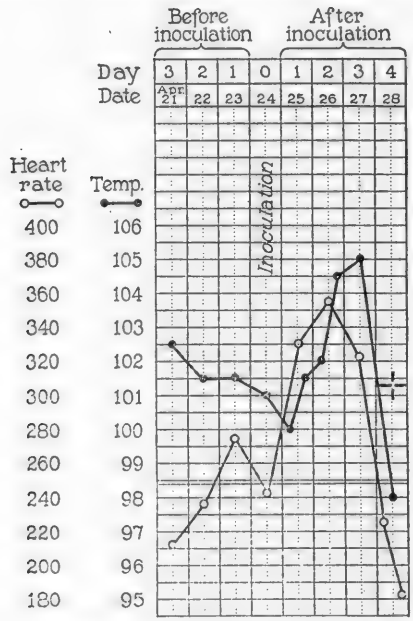
Electrocardiograms were taken either for 1, 3, or 4 days before the day of inoculation. Exception to this procedure occurred in Guinea



TEXT-FIG. 2. Temperature and heart rate curves of Guinea Pig 12, inoculated with *Leptospira icterohæmorrhagiæ*.

Pigs 4 and 6 and Monkey 3. The cardiac rate before inoculation of the guinea pigs was below 300 per minute three times in Guinea Pig 1, twice in Guinea Pig 2, and once in Guinea Pig 10. In the other nine guinea pigs it was always above 300, the range being from 302 to 371. In the monkeys the rate was above 400, except once in Monkey 2, and once in Monkey 5. The rate otherwise was above 400, the range being from 401 to 442.

In eleven animals there occurred a fairly consistent fall in rate either immediately after inoculation (Guinea Pigs 3, 7, and 9 and Monkey 2) (Text-fig. 1) or after a delay (Guinea Pigs 2, 4, 5, 8, and 12 and Monkeys 3 and 4) (Text-fig. 2). In several instances (Guinea Pigs 2, 4, 7, and 9) a rise occurred in the course of the disease. In each instance, however, there was a striking fall on the day of death, or during the days immediately preceding death. Guinea Pig 2



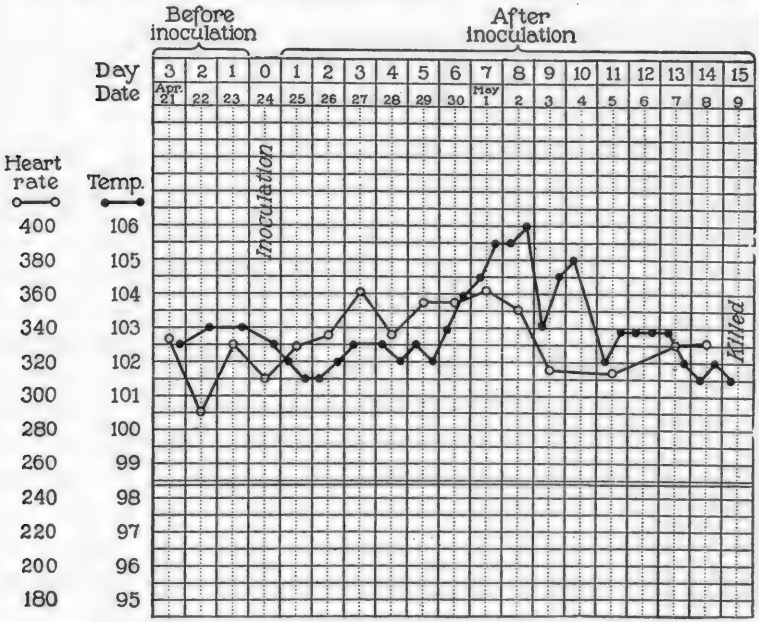
TEXT-FIG. 3. Temperature and heart rate curves of Guinea Pig 1, inoculated with *Leptospira icteroides* but with negative results.

appears to be an exception, but this is one of the animals in which low initial rates were recorded. The animals inoculated with *Leptospira icteroides* reacted, then, with a fall in rate. Those inoculated with *Leptospira icterohæmorrhagiæ* behaved in a similar manner (Table I).

In Guinea Pigs 1 (Text-fig. 3) and 6 the rates before inoculation were low, rose abruptly to high levels after inoculation, and then

fell. Guinea Pig 1 was inoculated with *Leptospira icteroides*, but died of a complicating peritonitis. Guinea Pig 6 ran an atypical course and died perhaps of a secondary infection.

This account leaves for consideration four animals, Monkeys 1 and 5 and Guinea Pigs 10 (Text-fig. 4) and 11. The monkeys did not develop disease of a nature that could be regarded as characteristic and are therefore offered as controls. They showed no striking



TEXT-FIG. 4. Temperature and heart rate curves of Guinea Pig 10, inoculated with *Leptospira icterohæmorrhagiæ* but with negative results.

change in rate. Like observations are afforded by Guinea Pigs 10 and 11. They were killed after 14 days. The course of the disease in them was mild and the lesions were atypical. They are, therefore, also to be regarded as controls.

Mechanism of the Heart Beat.—During the control period preceding inoculation in the *icterohæmorrhagiæ* group, a single animal, Guinea Pig 12, showed an irregularity of the cardiac mechanism on one

TABLE I.

Records of the Pulse Rate of Animals Inoculated with Leptospira icteroides and Leptospira icterohæmorrhagiæ.

Animal No.	Rate. Days before inoculation. 4	3	2	1	Average.	Day of inoculation.	Days after inoculation. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Fall in rate.*	Irregularity.	Result.
G. P.† 1		211	235	275	240	243	330	355	322	181	D.‡											−59	−	Died in 4 days. Negative: *L. icteroides* G§ 5. Peritonitis. (Text-fig. 3.)
" " 2		221	256	335	271	292	340	331	350	278	286	315	D.									+44	−	Died in 7 days. Positive: *L. icteroides* G§ 5. Fever 3rd, 4th, 5th, and 6th days; peritonitis.
" " 3	320	369	350	305	338	300	273	250	280	295	290	265	208	D.								−130	+	Died in 7 days. Typical: *L. icteroides* G§ 5. Fever 4th, 5th, and 6th days (Text-fig. 1).
" " 4						266	275	303	330		265	233	D.									−33	+	Died in 6 days. Typical: *L. icteroides* G§ 6. Fever 3rd, 4th, 5th, and 6th days.
" " 5	345	371	361	345	355	336	318	319	340	327	322	301	320	298	298	323	292	304				−51	−	Recovered in 10 days. Mild: *L. icteroides* G§ 6. Fever 5th day; mild.
" " 6						211	458	375	311	257	D.											+46	−	Died in 4 days. Atypical: *L. icteroides* G§ 6. Fever 4th day; perhaps secondary infection.

| | | |

TABLE I—*Concluded.*

Animal No.	Rate. Days before inoculation. 4	3	2	1	Average.	Day of inoculation.	Days after inoculation. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Fall in rate.	Irregularity.	Result.
G. P. 10		333	289	332	318	309	327	334	363	335	357	355	362	352	313		314		330	331		+13	−	Killed in 14 days. Probably negative or very mild: *L. icterohæmorrhagiæ* (French). Fever 7th to 10th days; no jaundice; no hemorrhage; no lesions (Text-fig. 4).
" " 11		360	340	360	353	361	360	376	363	350	371	367	362	342	306		320		327	328		−25	−	Killed in 14 days. Mild: *L. icterohæmorrhagiæ* (French). Fever 7th to 14th days; intercurrent infection; no jaundice; no hemorrhage; no lesions.
" " 12		358	343	347	349	345	360	370	363	344	370	363	374	356	340		254		276	238	D.	−111	+	Died in 14 days. Atypical: *L. icterohæmorrhagiæ* (French). Fever 7th to 10th days; incubation 6½ days (Text-fig. 2).
M. 5		436	436	372	415	432	415	431	392	370	D.											−45	−	Died in 4 days. Negative: *L. icterohæmorrhagiæ* (French). Doubtful; irregular fever; slight hemorrhage in lungs; no jaundice.

occasion. During the 14 days following, with two exceptions, curves were taken daily. A recurrence of the arrythmia was not seen. During the course of the disease nothing remarkable in the behavior of the heart was recorded, although curves of the animals in this group which died were taken on the day of death (Guinea Pig 12 and Monkey 5) and on the day preceding (Monkey 4).

Of the yellow fever group, no irregularity was seen in any animal in the period preceding inoculation. A single guinea pig, No. 7, on the 2nd day afterward, showed an arrythmia (premature ventricular contractions). It died 5 days later but the irregularity did not recur. In four other animals, irregularities took place (Guinea Pigs 3 and 4 and Monkeys 2 and 3), but in two of them (Guinea Pigs 3 and 4) they occurred on the day of death, indeed during the terminal period. At this period Guinea Pig 3 showed heart block (Fig. 1). Monkey 2 (Fig. 2) began to show changes in the electrocardiogram on the day before death. The alterations are not different from those which have been described in connection with the death of the heart in other diseases. There was slowing of the auricles and the whole heart, and increase in the length of the auriculoventricular interval in Guinea Pig 3 and Monkey 2 (Fig. 2) and lengthened conduction time leading to block in Guinea Pig 3. In only two of the animals, Monkeys 2 and 3 (Fig. 3), did changes occur before the day of death. In both the change was detectable 2 days before death. Both showed slowing of the rate and alteration in the form of the ventricular complex in the electrocardiogram. An S wave not previously present appeared, and the T wave increased in size (Fig. 2).

It may be said, then, that the mechanism of slowing in all cases until the day of death was of a simple variety—a slowing of the whole heart, depending on the sinus, the pacemaker of the heart. On the day of death, although increased length of the auriculoventricular interval occurred, it led to heart block only once. Changes in the form of the electrocardiogram were seen twice (Monkeys 2 and 3, Fig. 3) in the course of the disease 2 days before death. In curves that were obtained during death of the heart changes were seen which resemble those usually found at this time.

CONCLUSIONS.

1. Slowing of the heart occurred in monkeys and guinea pigs during the febrile period of the experimental infection due to *Leptospira icteroides*. A similar reaction took place in animals inoculated with *Leptospira icterohæmorrhagiæ*.

2. The mechanism of slowing was usually due to slowing of the whole heart.

3. Once incomplete heart block was seen. Changes in the ventricular complex occurred four times.

EXPLANATION OF PLATES.

In the curves divisions of the abscissæ equal 0.04 second, divisions of the ordinates 0.1 millivolt. The electrodes were placed on the right fore and the left hind legs.

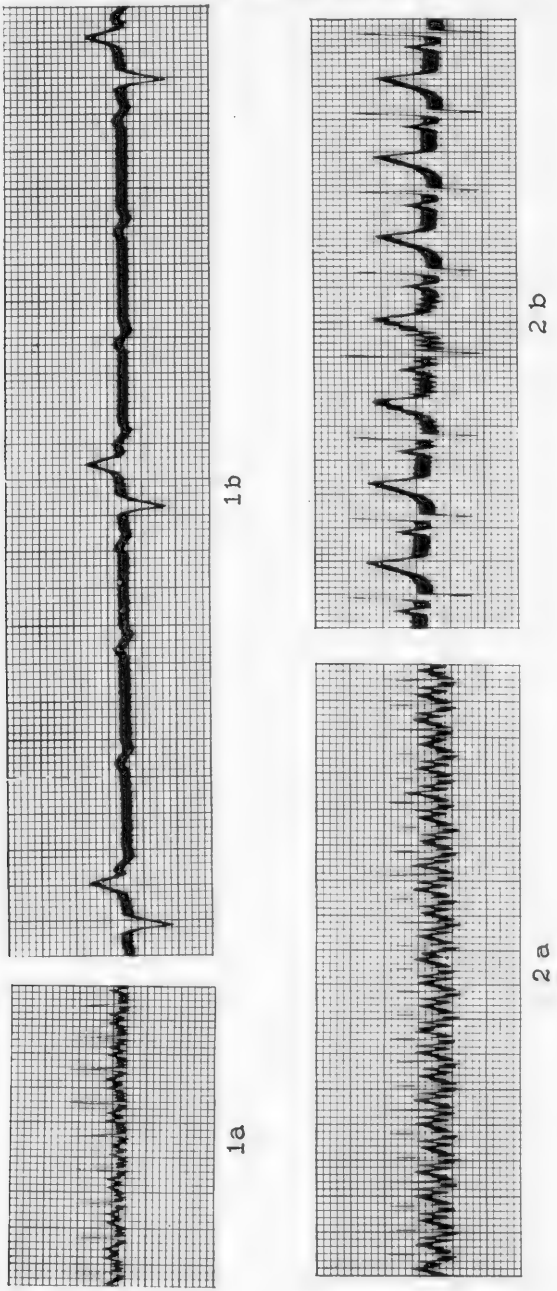
PLATE 85.

FIG. 1, *a* and *b*. Guinea Pig 3. (*a*) February 4, 1919. Control curve. (*b*) February 15. Auriculoventricular dissociation and a marked change in the ventricular complex are seen.

FIG. 2, *a* and *b*. Monkey 2. (*a*) April 21, 1919. Control curve. (*b*) April 30. Slowing, increase in height of R, S, and T waves, and increased auriculoventricular interval are seen.

PLATE 86.

FIG. 3, *a* to *g*. Monkey 3. (*a*) April 7, 1919. Control curve. (*b*) April 13. Day before death. S wave is increased. (*c*) April 14. Day of death. The R, S, and T waves are increased. The auriculoventricular interval is lengthened. (*d*, *e*, *f*, *g*) show increasing alteration in both auricular and ventricular parts of the curve. The ventricular alterations resemble those seen in dying hearts.



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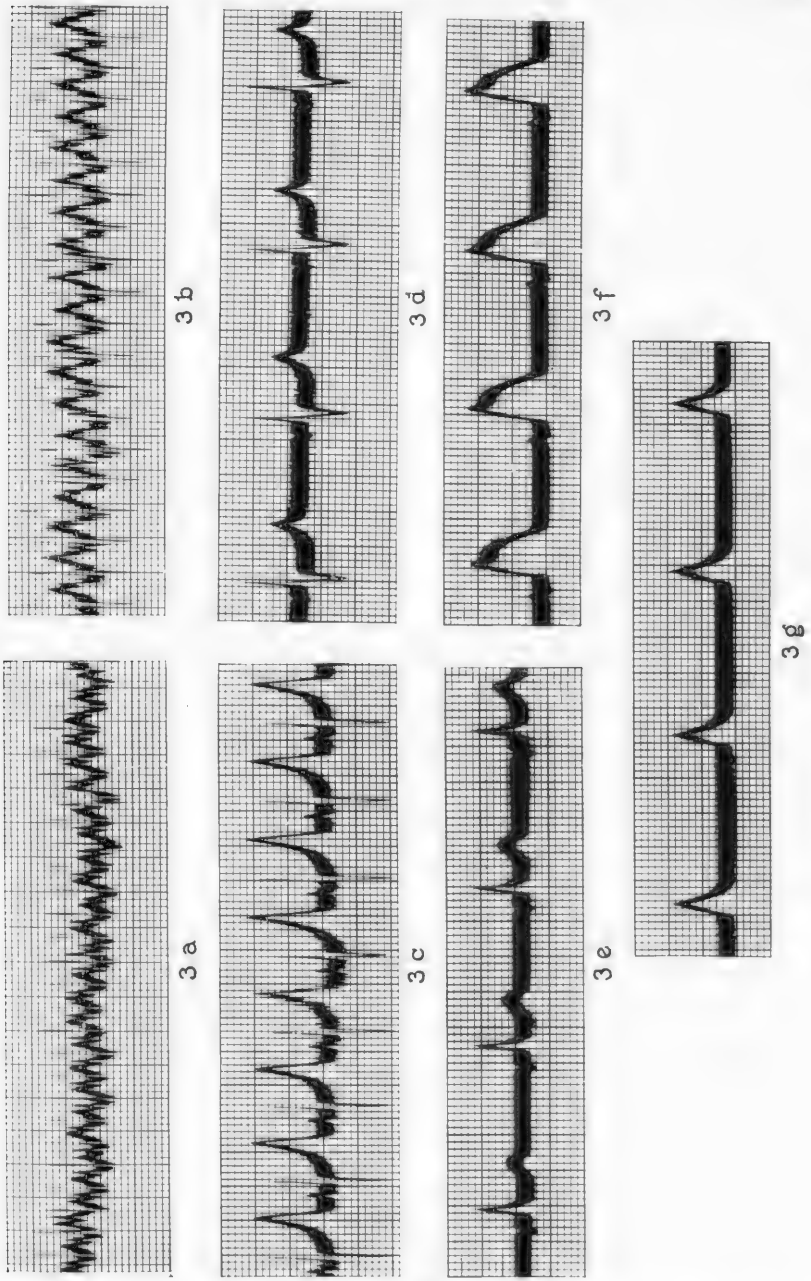

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CHEMOTHERAPEUTIC STUDIES WITH ETHYLHYDROCUPREINE HYDROCHLORIDE IN EXPERIMENTAL PNEUMOCOCCUS PLEURITIS.

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Plates 87 to 94.

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In previous experiments Kolmer and Idzumi (1) found that the subthecal injection of single doses of ethylhydrocupreine hydrochloride in amounts of 0.0005 gm. per kilo of weight had a distinctly beneficial effect on the course of acute suppurative meningitis in rabbits produced by Type I pneumococci of moderate virulence, when administered not later than 4 to 6 hours after infection; when given 24 hours or longer after the pneumococci, this effect was not apparent. In experiments consisting in the repeated subthecal injection of smaller doses of pneumococci followed in a few hours by ethylhydrocupreine, the protective and curative effects of the drug were more apparent.

Unquestionably, ethylhydrocupreine (optochin) hydrochloride shows marked pneumococcidal activity *in vitro* in menstrua of saline solution and in the presence of large amounts of protein, also some protective and curative properties in experimental pneumococcus bacteremias in mice. However, the drug is too toxic to permit the administration of sufficient amounts to individuals to influence materially the severity and mortality of lobar pneumonia, but experiments on pneumococcus meningitis have indicated that it may be possible to inject enough ethylhydrocupreine into closed sacs to raise the pneumococcidal activity of the exudates without danger of irritation of the serous membranes or the absorption of toxic amounts.

These results suggested similar experiments on acute suppurative pneumococcus pleuritis induced in guinea pigs and dogs as having a

possible value in the treatment of pneumococcus pleuritis with empyema developing during croupous pneumonia.

The purposes of the experiments were as follows: (1) to determine the pneumococcidal activity of ethylhydrocupreine hydrochloride in pleural pus; (2) to determine the toxic effects of ethylhydrocupreine injected into the pleural sacs; (3) to produce experimentally suppurative pneumococcus pleuritis; and (4) to determine the curative effects of ethylhydrocupreine in experimental suppurative pneumococcus pleuritis.

Pneumococcidal Activity of Ethylhydrocupreine in Pleural Pus.

Numerous experiments by Morgenroth and Levy (2), Moore (3), Solis-Cohen, Kolmer, Heist, and Steinfield (4), and others have

TABLE I.

Bactericidal Effect of Ethylhydrocupreine on Type I Pneumococci in Pleural Pus.

1 cc. of ethylhydrocupreine.	Pus.	Final dilution.	Results of subcultures after exposure at 37°C.			
			5 min.	30 min.	60 min.	120 min.
	cc.					
1:100	1	1:200	−*	−	−	−
1:500	1	1:1,000	+	−	−	−
1:1,000	1	1:2,000	+	−	−	−
1:2,000	1	1:4,000	+	+	+	+
1:3,000	1	1:6,000	+	+	+	+
Saline control.	1	−	+	+	+	+

* − indicates that subcultures were sterile; + indicates living pneumococci.

attested to the high bacteriostatic and bactericidal activity of ethylhydrocupreine for pneumococci *in vitro* in menstrua of saline solution and of serum. Germicidal tests *in vitro* by Kolmer and Idzumi (1) with purulent spinal fluids containing virulent Type I and Type II pneumococci showed ethylhydrocupreine to be bactericidal in a final dilution as high as 1:4,000 when equal parts of spinal fluid and drug solution were brought together for 1 hour at 37°C.

In the present study similar experiments *in vitro* with thick creamy pleural pus obtained from a patient and containing Type I pneumococci showed that with equal parts of pus and 1:1,000 solution of ethylhydrocupreine in saline solution complete sterilization occurs

after about 30 minutes in a water bath at 37°C. (Table I). Films of this pus showed the presence of enormous numbers of pneumococci, most of which were extracellular.

Portions of the same pus were heavily inoculated with a laboratory culture of *Staphylococcus aureus* and the bactericidal tests were repeated; mixtures of equal parts of pus and 1:100 solution of ethylhydrocupreine (final dilution 1:200) showed no appreciable diminution of the staphylococci after exposures as long as 2 hours, although the pneumococci were destroyed. Numerous experiments of this kind by the investigators previously mentioned have shown the high selective bactericidal activity of ethylhydrocupreine for pneumococci.

TABLE II.

Toxicity of Ethylhydrocupreine Hydrochloride Injected Intrapleurally in Guinea Pigs.

Weight of guinea pig.	Amount of 1:100 solution per 100 gm.	Amount of 1:100 solution injected.	Amount of drug per kilo.	Amount of 1:100 solution injected per kilo.	Result.
gm.	*cc.*	*cc.*	*gm.*	*cc.*	
360	1.0	3.6	0.100	10.0	Convulsions; died in 2½ min.
400	0.5	2.0	0.050	5.0	" " " 7 "
420	0.5	2.1	0.050	5.0	" " " few "
460	0.3	1.38	0.030	3.0	" " " " "
400	0.3	1.2	0.030	3.0	" " " " "
540	0.2	1.08	0.020	2.0	Nervous; coughing; lived.
400	0.2	0.8	0.020	2.0	Coughing; lived.
560	0.1	0.56	0.010	1.0	No symptoms; lived.
400	1.0 (water).	Control.	None.	10.0 (water).	Slight cough; "

Toxicity of Ethylhydrocupreine Injected into the Pleural Sacs.

Table II shows the results observed in an experiment consisting in the intrapleural injection of a 1:100 solution of ethylhydrocupreine into the pleural cavities of guinea pigs weighing from 350 to 400 gm.[1] The drug proved slightly more toxic when injected intrapleurally than when injected intravenously; this study has been extended with the use of other drugs and animals (5) with similar results.

[1] All injections were done under local anesthesia.

In each experiment the solutions were warmed and slowly injected; these are factors of importance in relation to toxicity. As shown in Table II the highest tolerated dose of ethylhydrocupreine hydrochloride for guinea pigs by the intrapleural route is between 0.2 and 0.3 cc. of a 1:100 solution per 100 gm. of weight, which is equivalent to 2 to 3 cc. or 0.02 to 0.03 gm. of the drug per kilo of body weight. In experiments on acute pleuritis 0.5 to 1 cc. of a 1:500 solution was injected into each pleural cavity of guinea pigs weighing from 350 to 500 gm. These amounts were two and a half to five times less than the toxic doses and produced no gross or histological evidences of irritation of the pleura as determined by necropsies and histological studies at varying intervals after the intrapleural injections (Fig. 1).

The slow injection of as much as 15 cc. per kilo of weight of a warm 1:500 solution of ethylhydrocupreine into the right pleural cavity of a normal dog produces no immediate symptoms, except possibly a few short coughs; this dose has produced no discernible evidences of toxicity. When examined at necropsy 24 to 72 hours after injection a small amount of fluid is found in the pleural cavity accompanied by slight hyperemic changes in the lung but no other gross changes. Histological examination usually reveals hyperemia of the lung with slight hyperemia and leucocytic infiltration of the pleura (Fig. 2). This amount of solution introduces as much as 0.030 gm. of the drug, corresponding to 900 cc., or 1.8 gm., per 60 kilos of weight and representing an amount near the highest tolerated dose.

In as far as volume of fluid injected into a pleural cavity is concerned, it would appear that guinea pigs and dogs show no immediate effects, except a few short coughs, or remote effects from the slow injection of as much as 5 to 10 cc. of warm sterile water or saline solution per kilo of weight; these amounts correspond to 300 to 600 cc. per 60 kilos, or the weight of a young adult person.

Experimental Pneumococcus Pleuritis.

Experiments were conducted with a Type I pneumococcus from the sputum of a case of croupous pneumonia of such virulence that 0.000001 cc. of a 24 hour dextrose blood broth culture was usually fatal for a white mouse about 36 hours after intraperitoneal injection.

For the purpose of producing experimental pleuritis, guinea pigs and dogs were chosen because of their higher natural immunity to the pneumococcus. Rabbits were liable to succumb with pneumococcus bacteremia after the intrapleural injection of amounts of pneumococcus culture too small for the production of well defined and extensive suppurative pleuritis. The majority of the experiments were conducted with guinea pigs.

Pleuritis in Guinea Pigs.—The intrapleural injection of guinea pigs with 0.5 cc. of 24 hour dextrose broth cultures of this strain usually killed in about 50 hours with extensive suppurative changes in both pleural sacs and the pericardium (Table III). The injections were

TABLE III.

Production of Pleuritis in Guinea Pigs by the Intrapleural Injection of 24 Hour Dextrose Broth Cultures of Virulent Type I Pneumococci.

Weight of guinea pig.	Amount of culture.	Duration of life.	Cultures of heart.	Result.
gm.	*cc.*	*hrs.*		
410	0.5	50	+*	Bilateral suppurative pleuritis and pericarditis.
300	1.0	65	+	" " " " "
380	1.0	24	+	" " " " "
360	1.0	48	+	" " " " "
380	2.0	50	+	" " " " "
430	2.0 (broth control).	Lived.	0	Necropsy not performed.

* + indicates pneumococci in films or cultures of blood or both; 0, not done.

made with aseptic technique in the anterior axillary line on the right side in the neighborhood of the sixth or seventh interspace, all injections being slowly given.

Necropsies usually disclosed pneumococci in the heart's blood and extensive suppurative changes in the pleuræ which were most marked on the right side but also involved the pleura on the left side and the external portions of the pericardium (Fig. 3). Each pleural sac usually showed the presence of a plastic exudate of a greenish yellow color; films showed large numbers of polymorphonuclear pus cells

and very large numbers of extracellular diplococci (Fig. 4). Sections of the visceral and parietal portions of the pleura showed the histological changes of acute suppurative pleuritis, with very large numbers of polymorphonuclear leucocytes and fibrin deposits (Figs. 5 to 9). As a rule, sections of the visceral portions of the pleura showed more extensive changes than sections of the parietal portions. Animals which survived for 5 days or were permitted to live for longer periods of time showed evidences of beginning organization of the inflammatory exudate (Fig. 7) or well marked fibrous tissue thickening of the visceral and parietal layers of the pleura, particularly of the former (Fig. 10).

Generally the lungs on both sides showed inflammatory changes. At necropsy portions were usually dark red in color and failed to collapse. Sections of these areas showed intense hyperemia and serous exudation (Figs. 11 and 12); occasionally the alveoli contained numerous polymorphonuclear leucocytes and alveolar epithelium. These changes were most marked just beneath the visceral pleura and in the areas of both lungs which showed the maximum macroscopic changes. Not infrequently the pleuritis was most severe over the anterior portions of both lungs and pericardium and at the bases of the lungs in proximity to the diaphragm.

Controls injected intrapleurally with relatively large amounts (up to 20 cc. per kilo) of sterile 0.1 per cent dextrose blood broth lived indefinitely. Necropsies performed at intervals never showed suppurative changes; at most small amounts of blood-stained serous fluid were found on the injected side after 24 hours. Animals necropsied several days after injection showed normal sacs, except occasionally for a few fine adhesions between the visceral and parietal portions of the pleura in the immediate neighborhood of the sites of injection. Since the broth produced at most a very slight localized pleuritis, we used 24 hour broth cultures of the pneumococcus instead of cocci grown on solid media or obtained by centrifugation of broth cultures and suspension in saline solution. The slight pyogenic activity of the broth alone was considered an aid in the production of a severe form of suppurative pleuritis.

Pleuritis in Dogs.—With dogs weighing about 5 kilos acute pleuritis terminating fatally in about 3 days was produced by injecting 15 cc.

of a 24 hour broth culture reinforced by washing into the broth the cocci from ten slants of 24 hour growths on blood agar in tubes of the usual size. Within 24 hours the animals were sick with increased rectal temperatures and leucocytosis. Necropsies on or about the 3rd day after injection showed large amounts of thin bloody exudate (210 cc. in one instance) which deposited a heavy creamy layer of pus upon standing. Films of this exudate showed polymorphonuclear leucocytes, erythrocytes, and large numbers of extracellular pneumococci. Cultures of the blood from the heart usually showed the presence of pneumococci. The pleuritis was generally much milder than that observed in guinea pigs; the pleural surfaces were dull red and covered with flecks of fibrinous exudate but were generally free of the massive exudates developing among guinea pigs. Furthermore, there was usually no involvement of the pleura on the opposite side or of the pericardium, except the portion in direct relation with the pleura on the right side.

Sections of the pleura and lung showed acute pleuritis with well marked pulmonary congestion and edema (Fig. 11) and infiltration of the pleura with polymorphonuclear leucocytes.

Influence of Intrapleural Injections of Ethylhydrocupreine upon Pneumococcus Pleuritis.

Treatment with Single Doses of Ethylhydrocupreine Injected into the Right Pleural Sac.—In these experiments guinea pigs weighing from 350 to 500 gm. were employed. Each animal was infected by injecting 1 cc. of a 24 hour dextrose broth culture into the right pleural sac. The ethylhydrocupreine[2] was prepared in a 1:500 dilution in sterile saline solution and used in a dose of 1 cc. for guinea pigs of the weight given above. This dose is approximately 2.5 cc. of the solution, or 0.005 gm. of the drug, per kilo of weight. The ethylhydrocupreine was warmed to 37°C. and slowly injected once into the right pleural sac immediately after inoculation and at intervals varying from 4 to 48 hours after infection. Controls with culture alone and drug alone were included in each experiment. Necropsies were conducted aseptically for the purpose of securing films and cultures of the heart's blood and exudates; sections of the visceral and parietal pleuræ and lungs of each animal were prepared.

[2] Prepared by Zimmer and Company, Frankfort-on-the-Main.

In the first experiments, in which ethylhydrocupreine was injected into the right pleural sac only, there was a slight influence upon the infection as determined by the duration of life of the experimental animals. All animals died and usually showed acute suppurative pleuritis on both sides, which involved the pericardium and resembled that of the untreated controls. The results of one of the experiments are shown in Table IV.

TABLE IV.

Effect upon Pneumococcus Pleuritis in Guinea Pigs of Injecting 1 Cc. of 1:500 Ethylhydrocupreine into the Right Pleural Sac at Varying Intervals after Infection.†*

Interval between time of infection and injection of drug.	Result.‡
hrs.	
Immediately.	Lived about 48 hrs.; acute suppurative bilateral pleuritis and pericarditis.
4	" " 36 " " " " " " "
24	" " 52 " " " " " " "
48	" " 72 " " " " " " "
Culture alone (control).	Died in 48 " " " " "
Drug alone (control).	Lived.

* This is a dose equivalent to about 2.5 cc. per kilo.

† Animals infected by injecting 1 cc. of a 24 hour broth culture into the right pleural sac.

‡ All animals except the drug control showed pneumococci in the heart's blood and in the pleural exudates.

Treatment with Single Doses of Ethylhydrocupreine Injected into Both Pleural Sacs.—On account of the development of pleuritis on the left side and of pericarditis following the injection of pneumococci into the right pleural sac, subsequent experiments with guinea pigs were performed in which one dose of 1 cc. of 1:500 ethylhydrocupreine was injected into each pleural sac immediately after infection and at intervals of 4 to 48 hours after infection. Controls with the culture alone and drug alone were included in each experiment.

The results of two experiments are shown in Tables V and VI. In nearly all instances the severity and extent of the local inflammatory

TABLE V.

Effect upon Pneumococcus Pleuritis in Guinea Pigs of Injecting 1 Cc. of 1:500 Ethylhydrocupreine into Each Pleural Sac at Varying Intervals after Infection.†*

Interval between time of infection and injection of drug.	Result.‡
hrs.	
Immediately.	Lived.
4	" 48 hrs.; acute bilateral suppurative pleuritis and pericarditis.
24	"
48	" about 49 hrs.;§ acute bilateral suppurative pleuritis and pericarditis.
Culture alone (control).	" " 48 " " " " " " " "
Drug alone (control).	"

* This is a dose equivalent to about 2.5 cc. per kilo in each sac.

† Animals infected by injecting 1 cc. of a 24 hour broth culture into the right pleural sac.

‡ All animals necropsied showed pneumococci in the heart's blood and in the pleural exudates.

§ The animal was dying when injected with optochin; it died within 1 hour after injection.

TABLE VI.

Effect upon Pneumococcus Pleuritis in Guinea Pigs of Injecting 1 Cc. of 1: 500 Ethylhydrocupreine into Each Pleural Sac at Varying Intervals after Infection.†*

Interval between time of infection and injection of drug.	Result.
hrs.	
Immediately.	Lived.
2	" 4 days; serous exudate with few plastic adhesions.
4	" 8 " no exudate; areas of consolidation in both lungs.
24	"
Culture alone (control).	" 2 days; acute bilateral suppurative pleuritis and pericarditis.
Drug alone (control).	"

* This is a dose equivalent to about 2.5 cc. per kilo in each sac.

† Animals infected by injecting 1 cc. of a 24 hour broth culture into the right pleural sac.

changes in the pleuræ and pericardium and the duration of life of the animals were greatly influenced by the drug when the latter was administered within 24 hours after infection (Figs. 13 and 14). Untreated controls always showed extensive pleuritis and pericarditis at the end of 24 hours and usually succumbed within 48 hours (Fig. 3). In practically all instances when drug immediately followed culture the animals showed no signs of illness. Some were necropsied at intervals for histological study and showed no pneumococci in the heart or pleural sacs and no gross evidences of infection (Fig. 15) although sections of the visceral pleura sometimes showed slight degrees of leucocytic infiltration.

When the drug was administered 2 to 24 hours after injection the results were more irregular; that is, sometimes an animal that received the drug 24 hours after infection lived longer and showed less inflammatory reaction than animals that received the drug 2 to 4 hours after infection. In the majority of animals, however, the drug appeared to modify the degree and extent of pleuritis and pericarditis, unless treatment was delayed too long; *i.e.*, if 48 hours were allowed to elapse between the time of intrapleural injection of 1 cc. of the culture and the injection of 1 cc. of 1:500 ethylhydrocupreine hydrochloride into each pleural sac. When treatment was delayed for 48 hours the guinea pigs were usually very sick and not infrequently died within a few minutes after the intrapleural injections of the drug.

Treatment with Multiple Doses of Ethylhydrocupreine Injected into Both Pleural Sacs.—Additional experiments were conducted in which daily injections of 0.5 cc. of culture were given into the right pleural sac of guinea pigs. Treatment began 4 hours after the second injection of culture and was repeated daily 4 hours after each injection of culture; it consisted in the injection of 0.5 cc. of 1:500 ethylhydrocupreine into each pleural sac. The object of these experiments was to produce a milder pleuritis permitting longer duration of life. The fact that the first treatment was delayed 24 hours allowed the development of acute pleuritis which was generally confined to the right side. The conditions occurring in pneumococcus pleuritis in man were more nearly approached by administering the drug 4 hours after the culture.

The results of one experiment are shown in Table VII. Untreated controls usually died within 4 or 5 days, while controls that received drug alone survived after multiple injections. The results were usually irregular but practically always showed a distinct curative influence of the drug. Animals either survived for several days after the death of untreated controls or lived until killed for the purpose of making gross and histological studies of the pleuræ.

TABLE VII.

Effect of Ethylhydrocupreine upon Pleuritis in Guinea Pigs.

0.5 cc. of culture injected into right pleural sac. 24 hours later 0.5 cc. of culture injected into right pleural sac and daily thereafter. 4 hours after second injection of cocci, 0.5 cc. of 1:500 ethylhydrocupreine injected into both sacs, a dose equivalent to about 2.5 cc. per kilo. Injections repeated 4 hours after each injection of cocci.

Weight of guinea pig.	No. of injections of culture.	No. of injections of drug.	Result.
gm.			
500	4	3	Lived.
460	4	3	Died 7 days after first dose of culture;* small area of plastic pleuritis on right side; no changes in left pleura.
470	4	None (control).	Died 4 days after first dose of culture;† very extensive bilateral pleuritis and pericarditis.
400	None (control).	3	Lived.

* No pneumococci in heart's blood or exudate.

† Pneumococci in heart's blood and exudates.

Similar results were observed with dogs weighing about 5 kilos. These animals were infected by injecting into the right pleural cavity 15 cc. of a 24 hour broth culture reinforced by the cocci removed from ten blood agar slant cultures. Treatment was begun 24 hours after infection by injecting 15 cc. of a warm 1:500 solution of ethylhydrocupreine into the right pleural cavity and was repeated daily until four to seven doses had been given.

In an experiment of this kind the untreated control animal died 72 hours after infection with pleuritis of the right side and acute hyperemia and serous exudation of the right lung (Fig. 11); pneumococci

were recovered by culture from the heart's blood and pleural exudate. The left lung and pleura were not involved.

The treated animal received six injections of ethylhydrocupreine and survived. 24 hours after infection and just before the first treatment was begun, the animal appeared sick and the leucocytes had increased from 11,600 to 17,400 per c.mm. of blood. 24 hours after the first treatment the leucocytes were 17,000 per c.mm. and gradually fell each day until 9,400 were reached on the 6th day. On this day the rectal temperature was 38°C. and treatment ceased. This animal was in normal clinical condition when necropsied; the lungs and pleura on both sides were normal, there were no exudates, and cultures of the heart's blood and contents of the pleural sacs were sterile.

Treatment with Single Doses of Ethylhydrocupreine, Sodium Oleate, and Boric Acid Injected into Both Pleural Sacs.—Since Lamar (6) found that in the presence of boric acid, which overcomes the inhibitory influence of proteins, sodium oleate in 0.5 to 1 per cent solution is

TABLE VIII.

*Effect upon Pneumococcus Pleuritis in Guinea Pigs of Injecting 0.5 Cc. of a Mixture Composed of Equal Parts of 1:100 Ethylhydrocupreine, 0.5 Per Cent Sodium Oleate, and 5 Per Cent Boric Acid into Each Pleural Sac at Varying Intervals after Infection.**

Interval between time of infection and injection of mixture.	Result.
hrs.	
Immediately.	Lived.
2	"
4	"
24	"
24	"
Culture alone (control).	" about 48 hrs.;† severe bilateral suppurative pleuritis and pericarditis.
Mixture alone (control).	Lived.

* Animals infected by injecting 1 cc. of a 24 hour broth culture into the right pleural sac.

† Pneumococci in heart's blood and exudates.

destructive for pneumococci and increases their susceptibility to the destructive influence of antipneumococcus serum, we have experimented with a mixture prepared of equal parts of 1:100 ethylhydrocupreine, 0.5 per cent sodium oleate, and 5 per cent boric acid. 0.5 cc. of this mixture was injected into each pleural sac; 1 cc. or more of the mixture injected into each sac proved toxic.

The results of an experiment are shown in Table VIII. Mixtures of sodium oleate and boric acid alone had little or no influence, but with the addition of ethylhydrocupreine the results were frequently very successful and decisive, animals surviving even when infected 24 hours before treatment was begun.

Treatment with Single Doses of Ox Bile Injected into Both Pleural Sacs.—Since ox bile causes lysis and destruction of pneumococci, experiments were undertaken[3] with injections of fresh sterile bile into both pleural sacs of guinea pigs at varying intervals after infection. Preliminary experiments showed that injections of 0.5 to 2 cc. of undiluted bile into each pleural cavity of guinea pigs weighing from 350

TABLE IX.

Effect upon Pneumococcus Pleuritis in Guinea Pigs of Injecting 0.2 Cc. of Sterile Ox Bile into Each Pleural Sac at Varying Intervals after Infection.†*

Interval between time of infection and injection of bile.	Result.
hrs.	
2	Lived 48 hrs.;‡ bilateral pleuritis with extensive serous effusion.
4	" 48 " ‡ " " " " " "
24	"
24	" 4 days; no exudate; adhesions on right side.
Culture alone (control).	" 48 hrs.;‡ severe bilateral suppurative pleuritis and pericarditis.
Bile alone (control).	"

* This is a dose equivalent to about 0.5 cc. per kilo.

† Animals were infected by injecting 1 cc. of a 24 hour broth culture into the right pleural sac.

‡ Pneumococci in heart's blood and serous exudates.

[3] These experiments were undertaken at the suggestion of Dr. Allen J. Smith.

to 400 gm. were toxic, the animals dying at once or surviving less than 24 hours. In these experiments undiluted bile in a dose of 0.2 cc. was injected into each pleural cavity; the controls survived this amount and showed no toxic effects.

The results of one experiment are shown in Table IX. Occasionally a treated animal survived longer than the untreated controls. Even when treated animals succumbed, the macroscopic lesions appeared less extensive and the exudates more serous and less purulent in character.

The experiments were interrupted at this point before treatment with mixtures of bile and ethylhydrocupreine could be tried. Bile alone appeared to exert some beneficial influence but our experiments in this direction have not been sufficiently numerous to warrant further statements.

DISCUSSION.

Chemotherapy in Infections of the Serous Cavities.

Undoubtedly ethylhydrocupreine hydrochloride has been proved by numerous investigators to show an extremely high and selective pneumococcidal activity *in vitro*, even in the presence of proteins, as in menstrua of serum and transudates. These investigations have also disclosed a certain degree of protective power of the base in oil when administered subcutaneously, or the soluble hydrochloride salt when administered intraperitoneally in mice infected with lethal amounts of pneumococcus culture.

Unfortunately, the drug has proved too toxic when given by mouth or by intramuscular injection in the treatment of lobar pneumonia in man, to permit the administration of sufficiently large amounts to influence materially the severity and mortality of the disease. The transient toxic effects observed have been such symptoms as tinnitus, deafness, amblyopia, or retinitis. The retinitis, or amaurosis, may, however, result in more or less permanent impairment of vision.

Moore and Chesney (7) have shown, however, that when 0.024 gm. of ethylhydrocupreine hydrochloride per kilo of body weight, or about 1.7 gm. per 70 kilos, are administered daily by mouth and when the intervals between doses are not longer than 3 hours, a specific pneumococcidal property may be imparted to the blood which is maintained more or less constantly for several days.

In the treatment of pneumococcus meningitis and pleuritis it would appear that sufficient amounts of the drug may be injected intrathecally or intrapleurally to exert considerable pneumococcidal activity without irritating these membranes or causing toxic effects. When the drug is administered by mouth, intramuscularly, or intravenously, it is so highly diluted in the blood that enough cannot be administered to raise the pneumococcidal activity of the blood sufficiently for pronounced curative effects without danger of toxicity, but in the subarachnoid and pleural sacs dilution is by spinal fluid or exudate alone and is much less than in the blood.

The experiments by Kolmer and Idzumi in the treatment of experimental pneumococcus meningitis with ethylhydrocupreine showed that in mixtures of equal parts of purulent spinal fluid and 1:1,000 to 1:2,000 solutions of ethylhydrocupreine complete destruction of very large numbers of virulent pneumococci occurred within 1 hour at 37°C. The intrathecal injection of 0.5 cc. of 1:500 and 1:1,000 solutions per kilo of body weight, when administered not later than 4 to 6 hours after the injection of culture, had a distinct beneficial effect on the course of experimental meningitis in rabbits produced by Type I pneumococci. The drug was probably diluted in the subarachnoid space by 1 or 2 cc. of spinal fluid per kilo, giving final dilutions of 1:2,000 to 1:5,000; these dilutions of drug, however, apparently showed some pneumococcidal activity even in the presence of inflammatory exudates, and were found to have no irritative effects for the meninges. Furthermore, since the amount of drug injected at one time was 0.5 cc. of a 1:500 dilution per kilo of weight, or 0.001 gm., it was less than the toxic dose by intrathecal injection; this permitted the administration of multiple doses without toxic effects.

Similar but more decisive results have been observed in the present experiments. Since pleural pus is ordinarily much richer in pus cells it has required stronger solutions of ethylhydrocupreine to effect complete destruction of large numbers of pneumococci; in our experiments equal parts of pus and 1:1,000 solutions of ethylhydrocupreine kept at 37°C. are not completely sterilized in less than ½ to 1 hour.

Guinea pigs and dogs, however, show no ill effects from the intrapleural injection of 1 cc. of 1:500 ethylhydrocupreine per kilo. The toxic dose for guinea pigs appears to be approximately 2 cc. of a 1:100 solution per kilo, or 0.02 gm. of drug, so that the doses ordinarily employed in our experiments (1 cc. of a 1:500 solution for guinea pigs 350 to 500 gm. in weight in one or both pleural sacs) were at least two and one-half to five times less than the toxic doses.

The experimental lesions in guinea pigs were very severe, consisting of acute suppurative pleuritis which involved both pleuræ and the external portions of the pericardium. The intrapleural administration of ethylhydrocupreine in a dose corresponding to about 2 cc. of a 1:500 dilution per kilo of weight in each cavity, a total of 0.008 gm. of drug, had a marked curative influence in the majority of instances unless treatment was delayed longer than 24 hours. Such a delay permitted the development of severe bilateral pleuritis, pericarditis, and pneumococcus bacteremia. Multiple injections of 1 cc. of a 1:500 solution per kilo into each pleural cavity had an even more pronounced effect upon less severe infections.

These results indicate the possibility of chemical sterilization of infected serous cavities with drugs showing a marked and selective bactericidal activity for the infecting bacterium in the presence of pus, as optochin or ethylhydrocupreine hydrochloride for the pneumococcus, even though sufficient amounts of the drug cannot be administered by mouth, intramuscularly, or intravenously to influence a localized inflammatory process. It is highly probable that some of the drug injected into the cavity is absorbed into the blood, where it exerts some restraining influence upon the multiplication of bacteria.

SUMMARY.

1. Solutions of ethylhydrocupreine (optochin) hydrochloride show a pronounced bactericidal activity for pneumococci in pleural pus.

2. The highest tolerated dose of ethylhydrocupreine hydrochloride for guinea pigs by intrapleural injection is about 0.2 to 0.3 cc. of a 1:100 solution per 100 gm. of weight, corresponding to 2 to 3 cc. of solution or 0.02 to 0.03 gm. of the drug per kilo of weight.

3. The injection of 1 cc. of a 1:500 solution of ethylhydrocupreine hydrochloride into each pleural cavity of a guinea pig weighing from 350 to 500 gm. produces no evidences of toxicity or irritation of the pleura.

4. The injection of 1 cc. of a 24 hour dextrose blood broth culture of virulent Type I pneumococci into the right pleural cavity of guinea pigs produces acute suppurative pleuritis on both sides associated with suppurative pericarditis, which generally terminates fatally within 72 hours with pneumococcus bacteremia.

5. The injection of 1 cc. of 1:500 solutions of ethylhydrocupreine hydrochloride into each pleural cavity of guinea pigs at varying intervals up to 24 hours after pleural infection has usually shown a marked curative influence. Similar results were observed with dogs.

6. The intrapleural injection of mixtures of ethylhydrocupreine, sodium oleate, and boric acid (Lamar) has also shown a decided curative effect in acute suppurative pneumococcus pleuritis of guinea pigs.

7. These and similar experiments on pneumococcus meningitis suggest that in chemotherapeutic investigations certain drugs may be injected into serous cavities in amounts exerting distinct bactericidal activity *in vivo* without producing local irritation or general toxic effects.

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5. Kolmer, J. A., Route of administration of drugs in relation to toxicity in chemotherapeutic investigations, *J. Pharmacol. and Exp. Therap.* (in press).

6. Lamar, R. V., Chemo-immunological studies on localized infections. First paper: Action on the pneumococcus and its experimental infections of combined sodium oleate and antipneumococcus serum, *J. Exp. Med.*, 1911, xiii, 1; Second paper: Lysis of the pneumococcus and hemolysis by certain fatty acids and their alkali soaps, 380; Third paper: Some further observations upon the action of certain soaps on the pneumococcus and its experimental infections, xiv, 256; Fourth paper: Experimental pneumococcic meningitis and its specific treatment, 1912, xvi, 581.
7. Moore, H. F., and Chesney, A. M., A study of ethylhydrocuprein (optochin) in the treatment of acute lobar pneumonia, *Arch. Int. Med.*, 1917, xix, 611.

EXPLANATION OF PLATES.

PLATE 87.

FIG. 1. Right lung and pleura of a guinea pig that received 1 cc. of 1:500 ethylhydrocupreine hydrochloride into each pleural cavity as a control on the animals shown in Figs. 12 and 13. This animal was living and well 7 days later when killed.

FIG. 2. Section of the right pleura and lung of a dog 4 days after the intrapleural injection of 15 cc. of a 1:500 solution of ethylhydrocupreine hydrochloride per kilo of weight.

PLATE 88.

FIG. 3. Acute bilateral suppurative pleuritis and pericarditis in a guinea pig 36 hours after the injection of pneumococcus culture into the right pleural cavity.

PLATE 89.

FIG. 4. Film of pleural exudate showing large numbers of extracellular pneumococci 48 hours after the intrapleural injection of pneumococcus culture.

FIG. 5. Acute purulent pleuritis and pneumonia. Section of the right pleura and lung of a guinea pig 36 hours after the intrapleural injection of pneumococcus culture.

PLATE 90.

FIG. 6. Acute purulent pleuritis of a guinea pig 48 hours after the intrapleural injection of pneumococcus culture. The exudate in the pleural space between the visceral and parietal layers is shown.

FIG. 7. Beginning organization of the exudate in acute pleuritis of a guinea pig 5 days after the intrapleural injection of pneumococcus culture.

PLATE 91.

FIG. 8. Acute purulent pleuritis in a guinea pig 48 hours after the intrapleural injection of pneumococcus culture.

FIG. 9. Section of the right pleura and lung of a guinea pig that succumbed 48 hours after the intrapleural injection of pneumococcus culture.

PLATE 92.

FIG. 10. Chronic fibrous (organized) pleuritis of a guinea pig following acute pneumococcus pleuritis.

FIG. 11. Edema, hyperemia, and leucocytic infiltration in the right lung of a dog 72 hours after the intrapleural injection of pneumococci; there was an accompanying acute fibrinous pleuritis.

PLATE 93.

FIG. 12. Lung of the animal shown in Fig. 9.

FIG. 13. Section of the right pleura and lung of a guinea pig that was infected at the same time and in the same manner as the animal shown in Figs. 9 and 12, but received 1 cc. of 1:500 ethylhydrocupreine into each pleural cavity 4 hours after infection. This animal was living and well 7 days later when killed.

FIG. 14. Section of the right pleura and lung of a guinea pig that was infected at the same time and in the same manner as the animal shown in Fig. 8, but received 1 cc. of a 1:500 solution of ethylhydrocupreine in each pleural cavity 24 hours later. The animal was living and well 5 days later when killed.

PLATE 94.

FIG. 15. Lungs, pleuræ, and pericardium of a guinea pig that was infected at the same time and in the same manner as the animal shown in Fig. 3 but received 1 cc. of a 1:500 solution of ethylhydrocupreine hydrochloride in each pleural cavity 4 hours after infection and two subsequent injections at daily intervals. This animal was living and well 5 days later when killed.

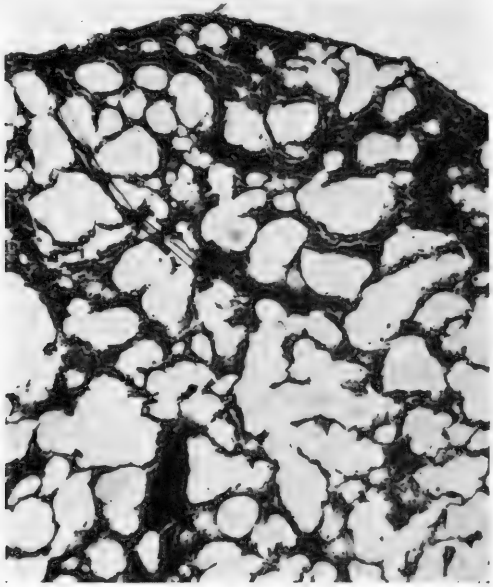

FIG. 1.

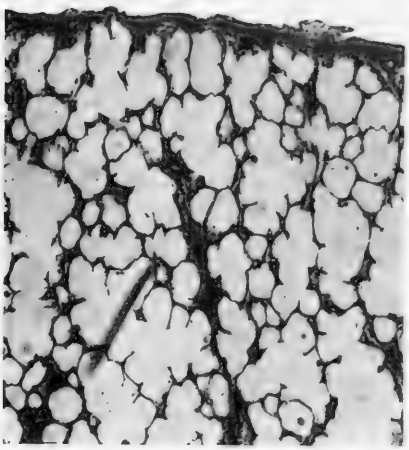

FIG. 2.

(Kolmer and Sands: Pneumococcus pleuritis.)

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FIG. 3.

(Kolmer and Sands: Pneumococcus pleuritis.)

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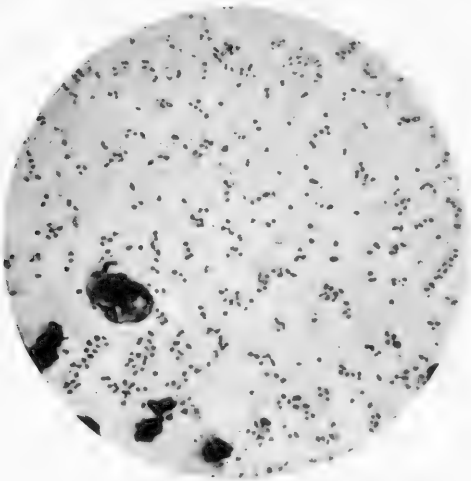

FIG. 4.

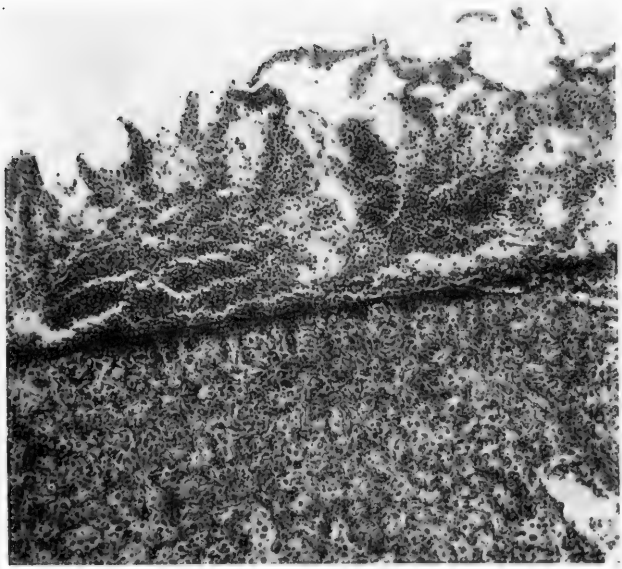

FIG. 5.

(Kolmer and Sands: Pneumococcus pleuritis.)

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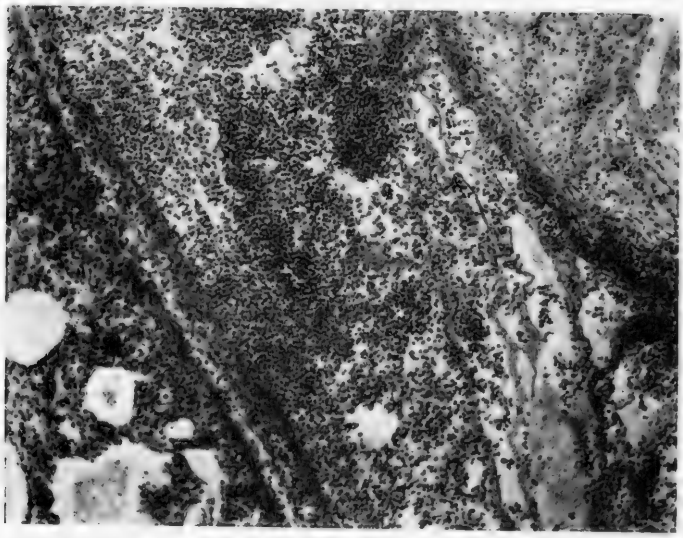

FIG. 6.

FIG. 7.

(Kolmer and Sands: Pneumococcus pleuritis.)

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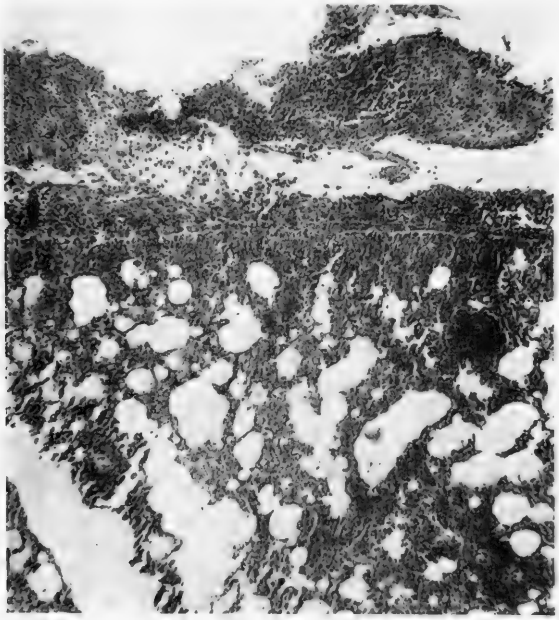

FIG. 8

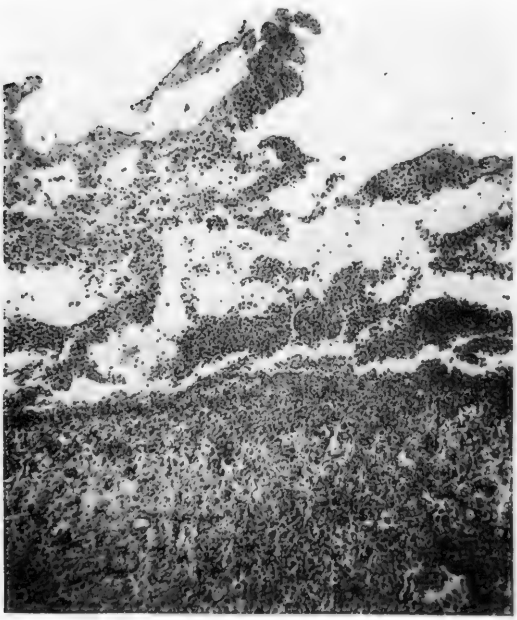

FIG. 9.

(Kolmer and Sands: Pneumococcus pleuritis.)

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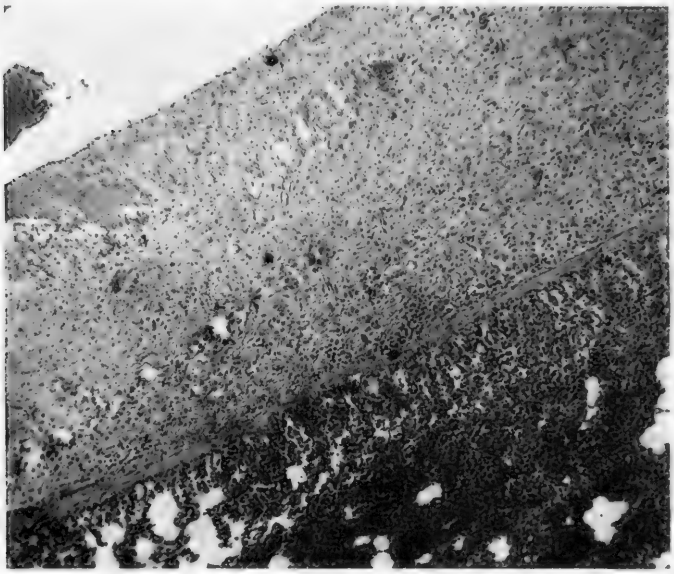

FIG. 10.

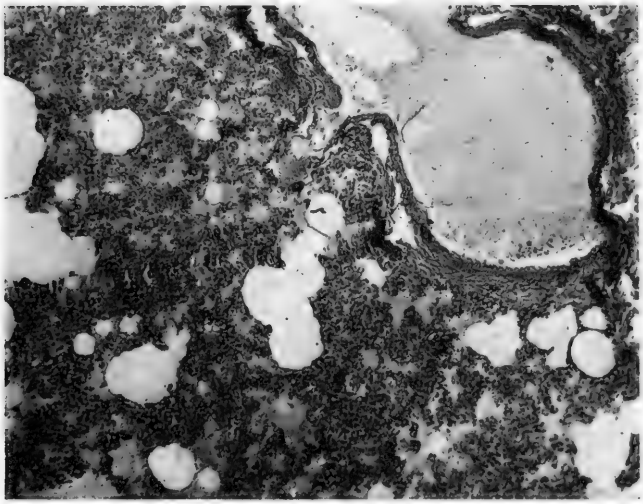

FIG. 11.

(Kolmer and Sands: Pneumococcus pleuritis.)

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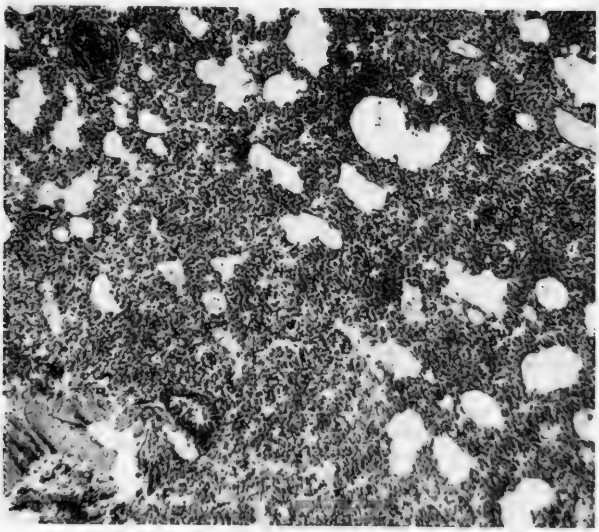

FIG. 12.

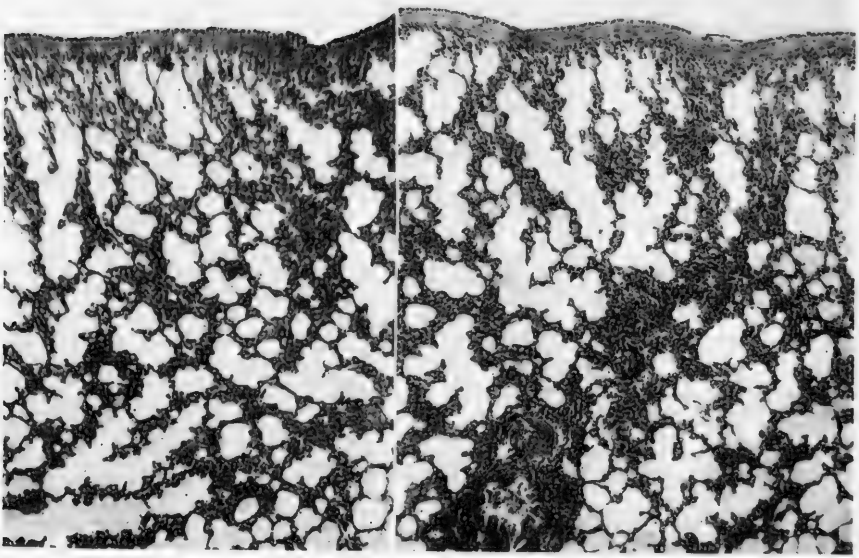

FIG. 13.

FIG. 14.

(Kolmer and Sands: Pneumococcus pleuritis.)

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FIG. 15.

(Kolmer and Sands: Pneumococcus pleuritis.)

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EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

IV. Anaerobic Cultivation.

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Plates 95 to 99.

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In a series of reports in this Journal[1, 2, 3] and in *The Journal of the American Medical Association*,[4, 5] we have described the changes in the blood and lungs of rabbits and guinea pigs which follow the intratracheal injection of unfiltered and filtered nasopharyngeal secretions, obtained within 36 hours after onset, from patients ill with uncomplicated epidemic influenza. The activity of the injected material was traced to the presence of a substance possessing characters which could only be attributed to a living agent, not, however, of the nature of ordinary bacteria. In the earlier reports[1, 4] we referred to experiments on the cultivation of this living agent by anaerobic methods, and recently[5] we described the nature of the characteristic, visible bodies usually found by cultural methods to be present in the nasopharyngeal secretions during the early hours of epidemic influenza in man and in the lung tissue of affected animals. The present paper describes these cultivation experiments in greater detail.

[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.
[2] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 361.
[3] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 373.
[4] Olitsky, P. K., and Gates, F. L., *J. Am. Med. Assn.*, 1920, lxxiv, 1497.
[5] Olitsky, P. K., and Gates, F. L., *J. Am. Med. Assn.*, 1921, lxxvi, 640.

Sources of Material.

The characteristic bodies were first observed in November, 1918, in strictly anaerobic cultures of the filtered nasopharyngeal secretions of an influenza patient in the early hours of the disease. Since that date successful cultivation experiments have been carried out with material ultimately derived from all of the seven cases of influenza from which active material was transmitted to animals. Numerous primary cultures, directly from human sources or from the lung tissues of affected animals, have been followed by hundreds of subplants, until at present certain strains are extant in the eighteenth generation.

The materials subjected to cultivation, essentially the same as those injected in the transmission experiments, were obtained from a variety of sources. During the two epidemic waves of influenza, that of 1918–19 and that of 1920, nasopharyngeal secretions were collected from patients with the uncomplicated disease, both in the early hours of the affection and in the later stages, including convalescence. During the first epidemic filtered and unfiltered material was also obtained from the lungs, at autopsy, of patients who had succumbed to secondary or concurrent pneumonias. In the course of the various series of transmission experiments with rabbits and guinea pigs, the lungs of affected animals were cultured both aerobically and anaerobically as a routine. Usually this material was not filtered. Sometimes the tissues were ground in saline solution and filtered in order to remove bacteria of the ordinary species. As a routine also, portions of the lungs of affected animals were preserved in sterile 50 per cent glycerol. These glycerolated specimens, after preservation for periods up to 14 months, were directly cultured or injected intratracheally into rabbits, from whose lungs cultures were made at autopsy.

The control materials for cultivation experiments were likewise similar to those used in control transmission experiments in animals. They consisted of unfiltered and filtered nasopharyngeal secretions from healthy persons during the two epidemic waves, the interepidemic recession, and the postepidemic period. Nasopharyngeal washings from persons suffering from acute coryza were also cultured

during the non-epidemic intervals. The normal lungs of uninoculated rabbits, the diseased lungs of stock rabbits who fell ill of other infections, the lungs of rabbits injected with control materials, and, finally, each of the various ingredients of the culture medium were subjected to anaerobic cultivation as controls.

Methods of Cultivation.

All the methods of anaerobic cultivation employed involved the use of fresh sterile kidney tissue in tubes or flasks of human ascitic fluid or ascitic fluid agar, usually under a vaseline seal.

In the early cultivation tests sterile paraffin oil was used as a seal instead of vaseline, and the tubes were incubated in an anaerobic jar set up as described by McIntosh and Fildes.[6] The decolorization of a tube of methylene blue in broth, included in the jar, indicated the establishment of anaerobic conditions. In later experiments the complete exclusion of oxygen by a vaseline seal permitted the rapid production and the maintenance of anaerobic conditions by the reducing substances in the medium.[7] The use of the anaerobic jar was, therefore, discontinued.

For primary isolations and routine cultivations the culture tubes were set up as follows: Relatively large pieces (0.6 to 0.8 gm.) of sterile normal rabbit kidney tissue were placed in test-tubes measuring 20 by 1.5 cm., one piece to each tube. Then 1 cc. of suspected fluid or 0.5 cm. of affected lung tissue was placed directly on the kidney tissue. These materials were covered with 8 to 10 cc. of sterile human ascitic fluid of a hydrogen ion concentration of 7.8 to 8. Ascitic fluids of a higher alkalinity were discarded. Sealed with 2 cc. of melted sterile vaseline and stoppered with cotton, the tubes were incubated at 37°C. for 8 to 12 days.

For special purposes this routine culture method was modified in two particulars. Occasionally tubes of a semisolid medium or mass cultures in flasks were employed, especially for subplants. The semisolid medium consisted of 1 part of beef infusion (2 per cent) agar, pH 7.4, to 2 parts of human ascitic fluid, mixed in a flask at 40°C.

[6] McIntosh, J., and Fildes, P., *Lancet*, 1916, i, 768.

[7] Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiii, 51.

and pipetted onto the kidney tissue and inoculum in the tall test-tubes. The medium for mass cultures was slightly modified from that described by Flexner, Noguchi, and Amoss[8] for the cultivation of the globoid bodies of poliomyelitis. In each Florence flask, of 50 cc. capacity, was placed one-fourth of a moderate sized rabbit kidney. The sections were cut across the entire kidney and placed with the cut surface parallel to the base of the flask. After inoculation the kidney was just covered (10 cc.) with the semisolid ascitic fluid medium described above and the agar was hardened by immersion of the flask for 15 minutes in cold water. Then the flask was filled to the neck with a mixture of 1 part of beef infusion broth, pH 7.4, and 2 parts of ascitic fluid. A seal of vaseline, 1 cm. deep, and a cotton stopper completed the preparation.

An advantage of the vaseline seal over the anaerobic jar was found in the ease with which specimens for examination or transplant could be obtained with a capillary pipette without exposure of the medium to the air. After puncture the seal was restored by gentle heat—just sufficient to melt a portion of the superposed vaseline.

All the earlier inoculations were made directly on the kidney tissue before the medium was added. More recently the incubation period has been shortened and growth facilitated by inoculating sterile preparations set up a day or two in advance, to permit the establishment of anaerobic conditions under the vaseline seal. The inoculum is placed in the vicinity of the kidney by means of a capillary pipette.

In primary cultivations and early subplants the suspected material was distributed among three to six culture tubes. Frequently only a few tubes of several in an initial cultivation showed growth. But apparent failure in the initial cultivation might be followed by success in a subplant. Hence at least two negative cultivations in succession were required before a culture series was discarded as negative.

Appearance of Positive Cultures.

The first evidence of the multiplication of a living substance in primary cultures in fluid medium was observed in the presence of a faint haze, first visible about the 5th day, extending upward about

[8] Flexner, S., Noguchi, H., and Amoss, H. L., *J. Exp. Med.*, 1915, xxi, 91.

1 cm. from the level of the kidney. This faint even cloud gradually became denser, reaching its maximum about the 8th day, when it approximated 3 cm. in depth. At the same time the clear supernatant fluid developed a characteristic very faint opalescence, often hardly discernible. On standing at room temperature the cloud gradually settled down, in the course of 2 weeks, to the region of the kidney tissue, leaving a clear supernatant fluid.

Initial cultures in a semisolid medium usually failed to show signs of growth. Subplants from the fluid medium developed slowly in semisolid cultures with the formation of almost microscopic colonies, too small to be defined with exactness. The evidence of growth was an even clouding of the medium extending to 3 to 2 cm. from the surface.

In mass cultures of well established strains a turbidity appearing throughout the semisolid layer about the 3rd day was followed by a diffuse clouding of the fluid portion by the 5th day. On standing at room temperature the cloud gradually subsided to the vicinity of the agar, forming a dense homogeneous nebulous layer on its surface (Fig. 1).

Morphology and Staining Reactions.

The greatest concentration of cultivable bodies for microscopic examination was obtained from the very bottom of the culture tubes. 0.3 to 0.4 cc. of the cloudy sediment was drawn up in a capillary pipette. After removing the adherent vaseline from the pipette with gauze the first two drops of fluid were discarded and subsequent drops were spread evenly on a slide in thick films. The films were then dried in an incubator at 37°C., fixed with gentle heat—three rapid passages through the Bunsen flame—and stained by the chosen method.

Of all the stains tried, well ripened Loeffler's alkaline methylene blue proved the most satisfactory. The preparation was flooded with the dye and steamed very gently over a flame for 2 minutes. The slide was thoroughly washed with running tap water and allowed to dry in the air.

Prepared in this manner, the films from typical cultures revealed minute bodies of regular morphology, stained a deep purple, and

clearly differentiated from the background of pale blue protein precipitate. The bodies were often exceedingly numerous, and with careful focusing, they stood out in sharp relief. Usually solitary, they were often found in diplo forms, and occasionally in short chains of three or four members. Clumps occurred also, especially in older cultures, the discrete definitely stained bodies forming masses from a blood platelet to a leucocyte in size. Viewed with the highest powers of the microscope, the bodies were seen to be two to three times longer in one direction than in the other. They were, therefore, bacilloid rather than coccoid. Thus they were differentiated sharply from the globoid bodies of poliomyelitis, which they approached in size. Their long axis measured 0.15 to 0.3 microns. Occasionally longer individuals were seen, but the organisms showed little tendency to pleomorphism and were characterized by uniformity in size and shape. Irregular staining reactions have not been encountered. No granules, clubs, spores, or involution forms have been seen. The bodies in one culture in its eighteenth generation are morphologically identical with those in the initial specimen (Figs. 2 to 5).

Occasionally unmistakable clumps or colonies developed in the region of the kidney (Fig. 5), but when few in number the bodies were sometimes obscured by protein or stain precipitates (Fig. 4). Under such circumstances, the unaccustomed eye made them out with difficulty. For this reason stains which showed any tendency to precipitation on the slide were found unsuitable for their demonstration. Thus, although the bodies were died lavender with Giemsa's, Wright's, and Manson's stains, the preparations were as a rule unsatisfactory. Carbolthionine (Nicolle), carbolfuchsin, steaming safranine, and steaming fuchsin were likewise unsuitable. All the strains examined decolorized uniformly by Gram's method. The counterstain, safranine, required steaming. The Gram-negative reaction was a constant feature of young and old cultures.

The dark-field microscope has not afforded a satisfactory method of examination on account of the similarity in size of the cultivable organism to the familiar dancing bodies of control preparations.

Filterability.

The cultivable bodies, even in remote generations, have been found to pass Berkefeld V and N candles. They are, therefore, to be classed among the filter passers.

Cultural and Biological Characters.

The strict anaerobic and nutritive requirements of the cultivable bodies have necessarily limited the study of their cultural reactions. Certain conclusions have been drawn from repeated experiments.

Final Hydrogen Ion Concentration of Cultures.—The incubation of sterile kidney tissue in ascitic fluid has been shown to change the hydrogen ion concentration of anaerobic tissue cultures toward the acid side.[7] In our experiments the final hydrogen ion concentration of growing cultures and of uninoculated controls was the same; namely, pH 7.4 to 7.8. The organisms failed to grow in media of a pH of 8 or 7, which appear to be the outside limits of the favorable range.

Action on Carbohydrates and Alcohols.—Growth took place in the presence of dextrose, maltose, lactose, saccharose, inulin, and mannitol. No observable amounts of acid or gas were produced. The media containing dextrose and maltose (1 per cent) showed a heavier cloud than usual, but this may or may not have corresponded to a more active multiplication of the organisms. No characteristic odor was detectable in any of the cultures.

Symbiosis.—In the course of the experiments certain culture tubes, inoculated with unfiltered lung tissue, yielded growths of ordinary bacteria in addition to the characteristic bodies seen in the original culture tubes and later demonstrated in pure culture with filtrates of the mixed material. It thus appears that this organism can develop in symbiosis with *Bacillus pfeifferi*, the pneumococcus, *Streptococcus hæmolyticus*, *Streptococcus viridans*, and staphylococci. In a few experiments deliberate mixtures of these bacteria with the influenzal bodies were cultivated, and the organisms were subsequently separated by plating and by filtration.

Resistance.—No growth has been obtained in subplants of cultures heated to 56°C. for ½ hour or longer. Exposure to chloroform vapor for 1 to 1½ hours apparently destroys the organism. Viable organisms have been found, however, in fluid, semisolid, and mass cultures kept at room temperature, 20–24°C. (68–76°F.) for periods up to 6 months.

In these respects the resistance of the cultivated bodies is similar to that of the active agent in glycerolated specimens of the lung tissue from affected rabbits.[2]

Enumeration of Positive Cultures and the Sources from Which They Were Obtained.

In the earliest experiments, before a precise technique for culturing and demonstrating the cultivable bodies was developed, it may be presumed that some active materials gave negative results. Hence the following enumeration of successful cultivations is of more significance from the positive than from the negative point of view, and cannot be regarded as an indication of the actual incidence of these cultivable bodies in epidemic influenza in man. This is particularly true in view of the fact that cultural experiments were not accepted as positive unless at least two generations of the cultivable bodies were obtained.

Cultivation experiments were attempted with the filtered nasopharyngeal washings of eleven patients with uncomplicated epidemic influenza during the first 36 hours of the illness. Of these cultivations, six gave positive results. Five strains were obtained from eight patients in the 1918–19 epidemic and one strain from three patients in the recurrence of 1920.

Material from twenty-eight other patients was cultured during the later stages of the disease—from the 48th hour to convalescence. Only one culture yielded a growth. This material was obtained 48 hours after onset from a patient who died 2 days later from a secondary pneumonia.

The filtered nasopharyngeal washings of four patients suffering from pneumonias secondary to epidemic influenza were apparently negative. No growth was obtained with filtrates of the lung

tissue, at autopsy, of two patients who succumbed to the secondary pneumonias.

Although the cultivation of these peculiar anaerobic bodies from the lung tissues of a large number of affected rabbits and guinea pigs in the transmission experiments already described[1, 2, 3] is presumptive evidence that this organism was probably the causative agent in the lesions produced, it is nevertheless important to correlate the presence of these organisms in the human nasal washings with the pathogenicity of these washings for rabbits.

The transmission experiments were initiated with the nasopharyngeal washings of seven patients, all in the first 36 hours of illness. Three of these specimens, filtered and cultivated, yielded strains of the characteristic organism. The specimens from three other patients failed to produce a growth. The seventh specimen was not cultivated. An eighth specimen, obtained early, appeared to be negative both in cultivation and animal transmission experiments. Three strains of the cultivable bodies were obtained from specimens of nasopharyngeal washings which were not used for transmission experiments in rabbits.

Strains were ultimately derived, however, from all seven of the patients enumerated above by cultivation of the lung tissues of rabbits and guinea pigs affected in animal transmissions of the active nasopharyngeal material.

Beside the unfiltered and the filtered nasopharyngeal washings and the fresh lung tissues of affected rabbits and guinea pigs, a third source of active material, pathogenic for rabbits, was rabbit lung tissue which had been preserved in sterile 50 per cent glycerol for periods up to 9 months.[2]

None of the specimens of glycerolated lung which were directly cultivated yielded growths of the specific organism. We have already reported[2] the activity of certain specimens of the glycerolated material in initiating characteristic lesions when injected intratracheally in rabbits. From the fresh lung tissues of these affected animals, or their successors in the line of animal passage, the anaerobic bodies were cultivated in a number of instances. In this way the primary cultivation of rabbits' lungs from eleven series of experiments in which the active material had previously been immersed in glycerol

for 5 days to 9 months, yielded three cultures of these bodies; in one instance the length of glycerolation was 5 days, in another 1 month, and in the third 9 months. The two original sources of active material used in the above eleven series were Case 16,[1] representing the first epidemic period, and Case 26,[1] representing the second.

Control Cultivation Experiments.

Control cultivation experiments were made directly with the unfiltered or filtered nasopharyngeal secretions of twenty patients free from influenza. Eight of these patients were suffering from an acute coryza in the early or late stages. The control materials were collected in the epidemic, interepidemic, and postepidemic periods. None of these specimens yielded the cultivable bodies found in six of the eleven early cases of influenza examined.

Control cultures of the following materials also uniformly failed to yield growth of these bodies: the lung tissues of six stock rabbits which died of accidental or epidemic infections such as snuffles or pneumonia; uninoculated tubes of 36 samples of human ascitic fluid and portions of all the rabbit kidneys used in the culture media; and the lung tissues of 60 rabbits, either normal or injected intratracheally with control materials in the course of the transmission experiments. The control materials injected in these rabbits included normal rabbit lung tissue, saline solution, human ascitic fluid, rabbit serum, and ordinary bacteria.

Inoculation of Rabbits and Guinea Pigs with the Cultivable Bodies.

For a study of the effects of culture injections on animals, mass cultures[9] were generally used. Because of pressure of other experi-

[9] Mass cultures were prepared for inoculation as follows: The vaseline seal was removed from the Florence flask with a sterile wire and the fluid part of the culture centrifuged for 20 minutes at 1,500 revolutions per minute or until the supernatant fluid was clear. The fluid was then decanted, and the small button-like deposit of the growth left at the bottom of the tube was resuspended in saline solution. The centrifugation was repeated, the clear, supernatant saline solution was removed, and the small amount of sediment was again suspended in 4 cc. of saline solution, of which 3 cc. were used for the experiment.

ments most of the animal inoculations had to be deferred so that they were finally done with well established cultures several generations removed from the original human source.

When this part of the work was undertaken there were available for study three separate strains of cultivable bodies, two from the first epidemic and one from the second. These three strains were represented by cultures derived from nine different sources. One, a human strain, was the seventeenth generation subplant of a culture of the filtered nasopharyngeal secretion of Case 17[1] of the first epidemic. The others were first to seventh generation cultures of the lungs of different rabbits which had been injected with glycerolated lung tissue from earlier animals in the transmission series. Four of these cultures were thus originally derived from Case 16 and three were from Case 17 of the first epidemic. One came originally from Case 26 of the second epidemic of 1920.

Of these nine cultures with which rabbit passages were again initiated, only one, derived from rabbit lung tissue, and then in the third generation, failed to produce the effects regarded as typical for the active material in the earlier transmission experiments. The results of the intratracheal injection of the growth of mass cultures, in doses of 3 cc. were so uniform and familiar that a common description will suffice for the entire series.

On examination 24 hours after inoculation, the rabbits showed a rise in temperature and usually a conjunctivitis, varying from simple injection of the palpebral conjunctiva to a marked injection of the palpebral and ocular conjunctivæ. These signs were accompanied by a definite and often marked leucopenia, the result of a depression of the mononuclear cells (Text-figs. 1 and 2). In the animals which were kept for observation, these conditions persisted for 2 to 3 days, when the animals returned to normal. When the rabbits were killed during the reaction, a characteristic pathological picture was revealed in the respiratory organs.

Only the respiratory organs were visibly affected. The lungs (Figs. 6 and 7) were voluminous with edema and emphysema. Numerous hemorrhages were to be seen on the surface, either diffuse or discrete, and often in the form of minute petechiæ. The pleuræ were not

involved. On section of the lungs, the cut surface dripped a frothy blood-stained fluid, evidence of a hemorrhagic edema. Hemorrhages similar to those which had reached the surface were scattered through the parenchyma. The trachea and bronchi showed a mucopurulent exudate covering an exfoliated and hemorrhagic epithelium.

Microscopic sections (Figs. 8 and 9) confirmed the gross observations. The hemorrhages, diffuse or discrete, were located in the interalveolar tissue, which was distended with edema and torn by emphysema. The interalveolar structures were also infiltrated to some degree with a cellular exudate consisting of mononuclear cells and some polymorphonuclear cells with large eosinophilic granules. Large cells of the respiratory epithelial type, probably desquamated bronchial epithelium, and numerous erythrocytes were seen in the parenchyma. No pneumonic consolidation was present.

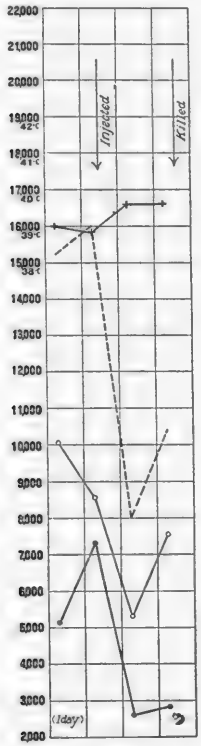
The bronchi showed thickened, hyperemic walls and their lumina were partly filled with erythrocytes, leucocytes, and fragments of exfoliated and necrotic epithelium. The lung capillaries were distended with blood.

As noted above, eight series of animal transmission experiments were initiated by the intratracheal injection in rabbits of pure cultures of the anaerobic bodies. From the lung tissues of rabbits in seven of these series the anaerobic bodies were recovered in pure culture.

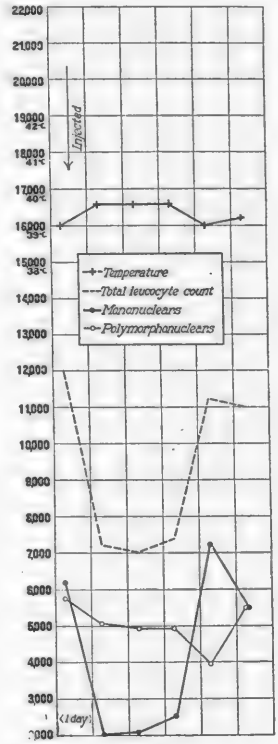
Both the strain obtained directly from the filtered nasal washings of Case 17, then in its seventeenth generation, and the strains derived from Cases 16, 17, and 26[1] after rabbit passage and glycerolation, produced the typical effects described above. The following protocols illustrate the similarity of the clinical effects produced by the human and the rabbit strains.

Protocol 1.—Preliminary observations on a rabbit for 2 days prior to inoculation gave the following results: temperature 39° and 38.9°C.; total leucocytes 15,200 and 16,000; mononuclear cells 5,168 and 7,360. Oct. 18, 1920. Inoculated intratracheally with 3 cc. of the growth of a second generation mass culture from the fifth rabbit passage of material from Case 16. Oct. 19. Temperature 39.6°C.; total leucocytes 8,000, of which 2,640 were mononuclears. Oct. 20. Temperature 39.6°C.; total leucocytes 10,400, of which 2,808 were mononuclears (Text-fig. 1). Rabbit killed. The lungs showed the hemorrhagic, edematous changes regarded as typical.

Another rabbit injected with the third generation mass culture of the same strain showed a similar picture of leucopenia and mononuclear depression. The rabbit was allowed to recover. This condition lasted for 3 days (Text-fig. 2).



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIG. 1. Effect on blood and temperature of the intratracheal injection of the cultivable bodies in a rabbit. The rise in temperature and the leucopenia due to mononuclear depression are noteworthy.

TEXT-FIG. 2. The same as Text-fig. 1 except that in this case the animal was allowed to recover. The persistence of the effects for 3 days is seen.

The next protocol is presented to show the effect of inoculation of a strain obtained by cultivating the nasopharyngeal secretions from a human case.

Protocol 2.—Jan. 6, 1921. A rabbit with normal temperature, 39°C., total leucocytes 10,400, of which 4,472 were mononuclears, was injected intratracheally with 3 cc. of the growth in the seventeenth generation mass culture of a strain from the filtered nasopharyngeal washings of Case 17. 24 hours later the animal showed a mild conjunctivitis, temperature 39.5°, total leucocytes 6,400, of which 1,152 were mononuclears. Jan. 8. The conjunctivitis was severe, total leucocytes 8,000, of which 2,400 were mononuclears. The lungs showed the lesions regarded as typical. The anaerobic bodies were recovered in pure culture from the lung tissue. The injection of this culture produced similar effects in a second rabbit, from the lung tissues of which the strain was again recovered.[10]

Several series of experiments were made with guinea pigs instead of rabbits. In this species the response to the intratracheal inoculation of cultures was similar to that obtained in rabbits, and from the affected lungs of the guinea pigs, the cultivable bodies were recovered.

Among the specimens of affected lung tissue preserved in 50 per cent glycerol were several which had come from rabbits injected with cultures of the anaerobic bodies. Although subsequent direct cultivations of these preserved specimens gave negative results, the same bodies were recovered from rabbit passages of the glycerolated material containing them. We thus have evidence that the bodies themselves do withstand glycerolation. The lungs from which these bodies were recovered by animal passage were immersed in glycerol for periods up to 4 months.

DISCUSSION.

These experiments seem definitely to connect the cultivable bodies with the clinical effects and lesions induced in rabbits and guinea pigs by the intratracheal injections of nasopharyngeal washings from patients with uncomplicated epidemic influenza.

From the lung tissues of such affected animals the morphologically and culturally characteristic bodies have been obtained in pure culture on special media by a strictly anaerobic technique. The bodies have been cultivated in successive generations without change in character. When injected intratracheally into rabbits and guinea

[10] The relation of ordinary bacteria in regard to their ability to produce concurrent or secondary infections in the presence of the cultivable bodies will form the basis of another paper.

pigs, they have given rise to pathological lesions in all respects similar to those from which they were obtained.

From the lesions the typical bodies have again been recovered in pure culture by the method employed for their primary isolation. Comparison of the strains thus derived from animal passages with those obtained directly from filtered human nasopharyngeal washings shows them to be identical in morphology and cultural characters. Finally, both the active material of the transmission experiments and the cultivable bodies obtained from similar sources withstand glycerolation and pass through Berkefeld V and N filters.

We feel, therefore, that the active material, pathogenic for rabbits and guinea pigs, found in the nasopharyngeal secretions of patients in the early hours of uncomplicated epidemic influenza has been identified in the anaerobic organism described in this paper.

It would, of course, be a simple matter to announce the inciting or etiological agent of epidemic influenza in man to be the minute, bacilloid organism here described. At present such a course does not seem desirable even though the clinical and pathological effects induced in the rabbit simulate so closely the phenomena found in epidemic influenza in man. Apparently we are at the threshold of our knowledge of a group or class of minute microorganisms which the anaerobic Smith-Noguchi technique has thrown open to exploitation. It seems wiser, therefore, to defer decision of the precise relation which the species described in this and previous communications bears to epidemic influenza until further experience is obtained.

In the meantime it is desirable to give the microorganism a name, and since a striking feature of its effect in rabbits is to diminish the resistance of the lungs to the action of ordinary pathogenic bacteria, as will be shown in a forthcoming paper, the name of *Bacterium pneumosintes* is proposed (from πνεύμων, lung, + σίντης, injurer, or devastator).

SUMMARY AND CONCLUSIONS.

From the filtered nasopharyngeal washings of patients in the first 36 hours of uncomplicated epidemic influenza and rarely in later stages of the disease, we have cultivated a minute bacilloid body,

Bacterium pneumosintes, 0.15 to 0.3 microns in length, of constant cultural characters and capable of indefinite propagation on artificial media. This organism, not of the nature of ordinary bacteria, was also recovered in pure culture from the unfiltered and filtered lung tissue of rabbits and guinea pigs inoculated with unfiltered and filtered nasopharyngeal washings of early influenza cases, both from the first epidemic of 1918–19 and from the second one of 1920. The organism grows only under strictly anaerobic conditions, passes Berkefeld V and N filters, and withstands the action of sterile 50 per cent glycerol for a period of months.

It has been recovered from cultures contaminated with a variety of ordinary bacteria such as *Bacillus pfeifferi*, pneumococci, streptococci, and staphylococci, and has been experimentally cultivated in symbiosis with them.

Similar cultivation of control materials uniformly failed to yield growths of this organism. The materials tested consisted of the unfiltered and filtered nasopharyngeal washings of persons free from influenza, some of whom were suffering from acute coryza, the lung tissue of normal rabbits and of rabbits with bacterial respiratory infections, and the uninoculated media.

The intratracheal injection in rabbits and guinea pigs of mass cultures of this organism has induced effects on the blood and lungs of these animals which are not to be distinguished from those obtained with the nasopharyngeal secretions of patients in the early hours of epidemic influenza. From the pulmonary lesions thus induced the same organism has been recovered in pure culture, and has been found to cause similar lesions on subsequent animal passage. Its pathogenicity is not lost by prolonged artificial cultivation.

Our experiments indicate that the cultivable bodies obtained directly from human nasopharyngeal washings and from affected rabbit lungs are strains of the same organism. This organism appears to be the source of the reactions which occur in experimental animals —rabbits and guinea pigs—as a result of the intratracheal injection of nasopharyngeal washings obtained during the early hours of uncomplicated epidemic influenza in man.

EXPLANATION OF PLATES.

PLATE 95.

FIG. 1. The construction of mass culture medium, to the left, and the growth of the cultivable bodies therein, to the right. The growth is derived from a rabbit's lungs after 8 days incubation. The turbidity of the semisolid and the haziness of the fluid layers are noteworthy. Actual size.

PLATE 96.

FIG. 2. The cultivable bodies in the eighth generation. Culture obtained from a rabbit's lung into which was injected material, after 9 months immersion in glycerol, originally derived from the filtered nasopharyngeal secretions from a case of epidemic influenza. Stained with ripened Loeffler's alkaline methylene blue. × 1,000.

FIG. 3. The bodies in Fig. 2 highly magnified. Stained similarly. × 3,000.

FIG. 4. Comparative size of the cultivable bodies. An aerobic streptococcus and a chain of poliomyelitis globoid bodies, indicated by an arrow, have been superimposed. The cultivable bodies are very minute, uniform, and lightly stained, and are to be differentiated from the small irregular masses, deeply stained, which are protein precipitates. All × 1,000.

FIG. 5. The cultivable bodies in colony formation. × 1,000.

PLATE 97.

FIG. 6. The gross lesions of the lungs of a rabbit inoculated intratracheally with the first generation of the cultivable bodies. The hemorrhages, edema, emphysema, and absence of pneumonic consolidation are noteworthy. Natural size.

FIG. 7. These lungs were obtained from a rabbit which was inoculated with the lungs pictured in Fig. 6. The more intense lesions of this second passage of the cultivable bodies are seen, especially the hemorrhagic edema of the right lung. Natural size.

PLATE 98.

FIG. 8. Microscopic section of the lung lesions in a rabbit, caused by injecting the cultivable bodies intratracheally. The edema, the emphysema, the hemorrhages, and the cellular exudate are noteworthy. × 240.

PLATE 99.

FIG. 9. Another field of the same section shown in Fig. 8, demonstrating particularly the bronchial lesions. The necrotic and exfoliated bronchial epithelium and the edema of its walls are to be seen. The general edema and the vessel filled with blood may be observed. × 240.

FIG. 1.

(Olitsky and Gates: Nasopharyngeal secretions from influenza. IV.)

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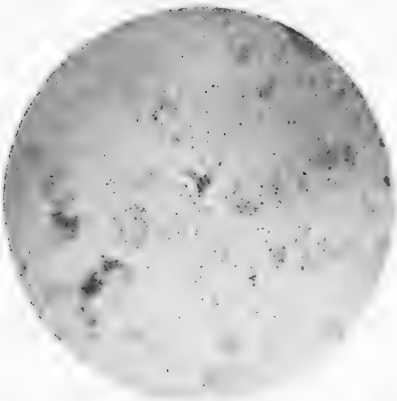

FIG. 2.

FIG. 3.

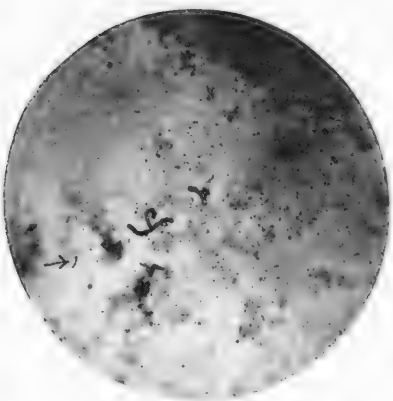

FIG. 4.

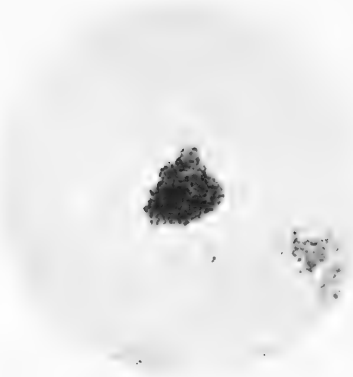

FIG. 5.

(Olitsky and Gates: Nasopharyngeal secretions from influenza. IV.)

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FIG. 6.

FIG. 7.

(Olitsky and Gates: Nasopharyngeal secretions from influenza. IV.)

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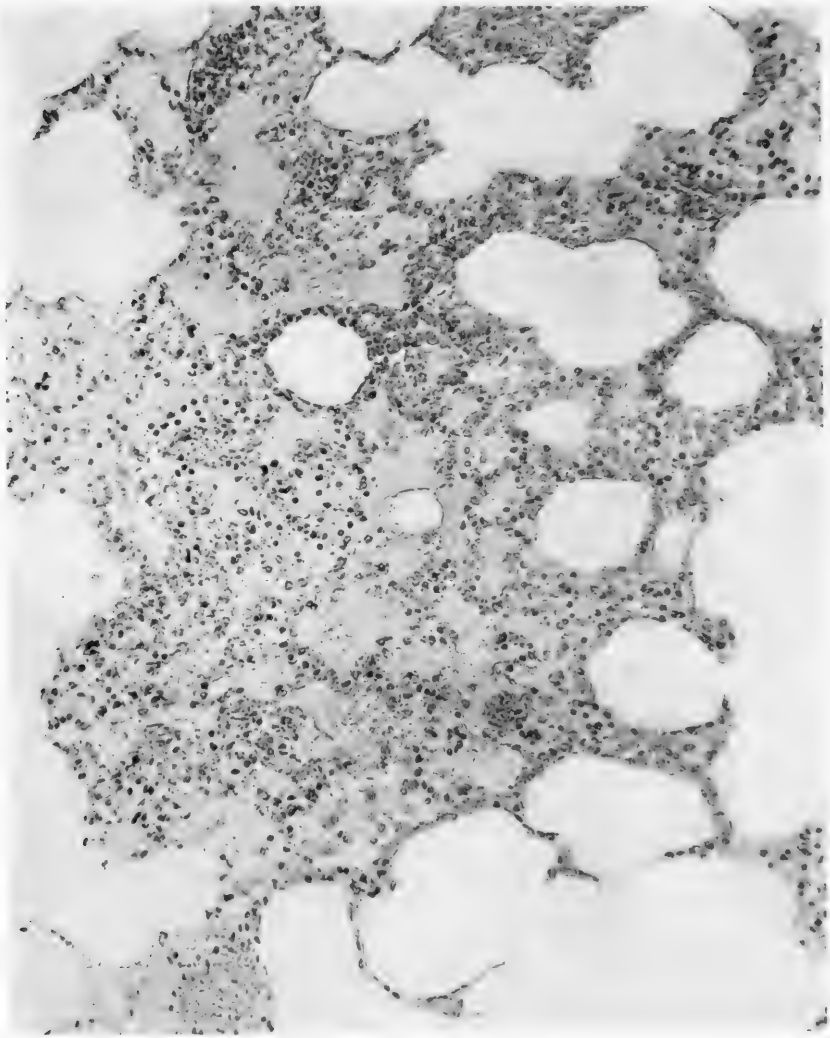

FIG. 8.

(Olitsky and Gates: Nasopharyngeal secretions from influenza. IV.)

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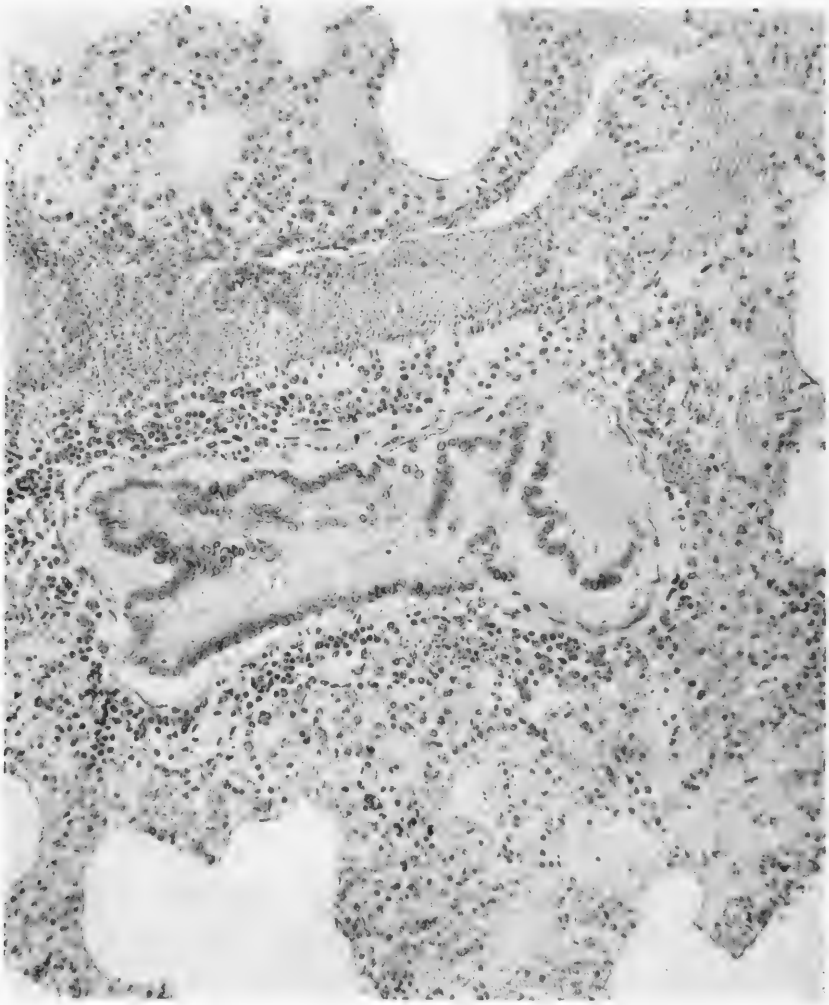

FIG. 9.

(Olitsky and Gates: Nasopharyngeal secretions from influenza. IV.)

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THE BILIARY OBSTRUCTION REQUIRED TO PRODUCE JAUNDICE.

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In a recent paper from this laboratory[1] observations were reported proving that a part of the rabbit's liver can function for the whole as regards bile elimination. A good instance in point is furnished by an experiment performed with another end in view. In a series of rabbits the ducts from three-fourths of the hepatic tissue were ligated and the portal branch to the remaining fourth was tied at the same operation, thus increasing the portal flow and by consequence bile formation in the mass with obstructed ducts. Under such circumstances the entire burden of bile elimination was laid on a mere quarter of the parenchyma, and this supplied with blood only by the hepatic artery, a source furnishing approximately two-fifths of the normal quantity if one may judge from data obtained in dogs.[2] Yet the rabbits remained unjaundiced and healthy. More recent observations upon dogs have convinced us that the experiment will yield a similar result with them. And it has been a surprise to discover on a search of the literature that no general recognition exists of the large margin of safety of the liver in bile elimination. On the contrary, frequent categorical statements may be found to the effect that, in man at least, jaundice is often caused by lesions affecting only a small proportion of the hepatic parenchyma.

Quincke and Hoppe-Seyler[3] state that transient obstruction of a small duct branch may bring about the absorption of sufficient bile to lead to a clinical

[1] Rous, P., and Larimore, L. D., *J. Exp. Med.*, 1920, xxxi, 609.

[2] Macleod, J. J. R., and Pearce, R. G., *Am. J. Physiol.*, 1914, xxxv, 87.

[3] Quincke, H. I., and Hoppe-Seyler, G., in Nothnagel, N., Specielle Pathologie und Therapie, Vienna and Leipsic, 2nd edition, 1912, xviii.

jaundice. The obstruction may be difficult to find at autopsy and the small ducts should be carefully searched for it. According to Eppinger[4] icterus is roughly proportional in intensity to the size of the occluded ducts, and localized inflammatory processes may cause it. Rolleston[5] believes that it follows the occlusion of one branch of the hepatic duct although the other continues to pour bile into the intestine; but not so according to Naunyn[6] who asserts that icterus under these circumstances fails to develop save when there is a complicating *Cholangie.* Krehl[7] holds that icterus occurs in many diseases through local stagnation and resorption. Recently van der Bergh and Snapper[8] have called attention to the fact that intrahepatic tumors of considerable size, and manifestly occluding many ducts, are often unaccompanied by jaundice, whence it follows in their opinion that local lesions in general must frequently exist without causing an accumulation of bile pigment in the organism.

The divergence of opinion illustrated by these specimen citations is obviously the result, first, of a lack of experimental evidence on the essential point at issue—the ability of a part of the liver to act for the whole as concerns bile elimination—and, second, of conclusions from clinical instances complicated by many factors.

For the work here to be described dogs and monkeys have been employed. Rabbits could not be used because their bile pigment fails to react satisfactorily to the ordinary tests.

Criteria of Bile Retention.

The clinician as a rule is first apprized of deficient bile elimination in his patient by the development of bilirubinuria with or without a tissue icterus. As our prime aim has been to determine how much biliary obstruction may exist without clinical manifestations, the tests in current use have been adopted for the work.

These tests as applied to the blood are very unsatisfactory. The Gmelin reaction with blood serum, so strongly advocated by French workers,[9] is yielded only when bilirubinemia is obvious to the eye, while furthermore lutein gives a

[4] Eppinger, H., *Ergebn. inn. Med.*, 1908, i, 107.

[5] Rolleston, H. D., Diseases of the liver, London, 2nd edition, 1912.

[6] Naunyn, B., *Mitt. Grenzgeb. Med. u. Chir.*, 1919, xxxi, 537.

[7] Krehl, L., Pathologische Physiologie, Leipsic, 9th edition, 1918, 571.

[8] van der Bergh, A. A. H., and Snapper, J., *Berl. klin. Woch.*, 1914, i, 1109.

[9] Gilbert, A., Herscher, M., and Posternak, S., *Compt. rend. Soc. biol.*, 1903, lv, 530; 1905, lvii, 250.

positive reaction; and the intensity of color of the plasma itself, save in outspoken cases, is trustworthy only in the absence of hemolysis and of extraneous pigments such as carotin. The reactions of Obermeyer and Popper[10] have proved in our hands little more sensitive than that of Gmelin. The diazo test recently advocated by van der Bergh and Snapper[11] is delicate, and promises to be of great clinical value, but its use has been limited as yet and it is not entirely specific.[12] We have employed it in the present work in its negative aspect, as the criterion wherewith to rule out bilirubinemia, for which it would seem highly suitable. None of many dog sera obviously stained with bile has failed to give the test, and the unstained sera have regularly proved negative.

The diazo reagent is a mixture, made fresh each day, of the following stock solutions. (*a*) 5 gm. of sulfanilic acid and 50 cc. of hydrochloric acid in 1,000 cc. of distilled water. (*b*) 0.5 per cent solution of sodium nitrite in distilled water. For use 1 part of (*b*) is added to 50 of (*a*).

The oxalated or citrated plasma to be tested is first shaken briefly with two volumes of 95 to 96 per cent alcohol and centrifuged to throw out the precipitate that forms. The supernatant fluid will now contain all of the bilirubin unless the plasma held very large quantities, in which case some will be carried down with the precipitate and must be extracted with 64 per cent alcohol if a quantitative result is to be obtained. On the addition of the diazo reagent to the fluid containing bilirubin, in the proportion of 1 part to 4, the beautiful violet tint of azobilirubin appears after a few minutes. To determine its amount a colorimeter is used and a solution of pure bilirubin in chloroform (5 mg. per 100 cc.) to give with the reagent a standard tint.

The Gmelin reaction has been used to detect bilirubinuria, and to quantitate it Hooper and Whipple's[13] modification of Salkowski's method has been employed as routine. The sodium nitrite-nitric acid solution recommended by Gilbert, Herscher, and Posternak[9] has been found to yield a better Gmelin response than the ordinary fuming nitric acid.

In contrast with the tests just mentioned, those for bile salts in the urine are none of them satisfactory. Pettenkofer's reaction was employed for one series of dogs, but Hay's sulfur reaction was adopted for most of the animals as less open to technical error and relatively specific.[14] Attempts to detect cholates in the blood were after many trials abandoned. The method recently described by Hoover and Blankenhorn[15] whereby the salts are separated out by dialysis and

[10] Obermeyer, F., and Popper, H., *Wien. med. Woch.*, 1910, lx, 2592.

[11] van der Bergh, A. A. H., and Snapper, J., *Deutsch. Arch. klin. Med.*, 1913, cx, 540.

[12] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497.

[13] Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916, xl, 332.

[14] Lyon-Caen, L., *J. physiol. et path. gén.*, 1910, xii, 526.

[15] Hoover, C. F., and Blankenhorn, M. A., *Arch. Int. Med.*, 1916, xviii, 289.

TABLE I.

Bilirubinuria in the Absence of Bilirubinemia in Dogs.

Normal dogs.				Operated dogs.				
Dog No.	Procedure.	Urine.	Blood.	Dog No.	Operation.	Time of observation.	Urine.	Blood.
16	Normal dog. Fasting on 6th and 9th to 12th days inclusive.	Bile pigment present on 6th and 8th to 14th days inclusive.	Negative.	19	All ducts ligated but those to lateral liver mass; 71 per cent of liver obstructed.	Before operation.	Bile pigment present for 3 days.	Negative.
						After operation.	Bile pigment present on 1st to 8th, and on the 10th day after operation.	Pigment in plasma on 6th, 7th, and 8th days after operation.
17	Normal dog. Fasting on 6th and 9th to 14th days inclusive.	Bile pigment present on 6th, 7th, 9th, and 11th days.	"	8	Ducts to lateral mass left free; 71 per cent of liver obstructed.	Before operation.	Bile pigment present for 5 days.	Negative.
						After operation.	Bile pigment present on 2nd, 3rd, and 12th days.	"
18	Normal dog. Fasting on 6th day only.	Bile pigment present on 6th and 7th days.	"	10	Ducts to left lateral lobe left free; 72.4 per cent of liver obstructed.	Before operation.	Negative.	"
						After operation.	Bile pigment present on 4th, 5th, 10th, and 12th days.	"

				12	Duct to right lateral lobe left free; 82 per cent of liver obstructed.	Before operation. After operation.	Negative. Bile pigment present on 4th, 9th, and 11th days.	Negative. " until 11th day, then faintly positive.
				11	Duct to right lateral lobe left free; 82 per cent of liver obstructed.	Before operation. After operation.	Negative. Bile pigment present on 6th day.	Negative. "

concentrated failed regularly in our hands to give positive results with the plasmas of jaundiced dogs excreting cholates through the kidneys. This may have been due to the lack in the dog of a renal threshold for bile salts such as in man leads to their accumulation in the circulation.

The Renal Threshold for Bilirubin.

In man, as is well known, the normal blood plasma has a yellow color owing to the presence of bilirubin; and a considerable increase in the pigment may occur without the passage of any into the urine, much less the appearance of a tissue icterus.[15] According to van der Bergh and Snapper[11,16] the plasma of healthy dogs is colorless and this has been our own finding except in animals with "physiological jaundice" induced by fasting. The plasma of normal monkeys is also in our experience practically colorless and fails to give the diazo reaction. We have further noted that in both dogs and monkeys bilirubinemia never occurs without bilirubinuria, while in dogs, at least, bile pigment is frequently to be found in the urine when it cannot be demonstrated in the circulation (Table I).

The state of the urine, then, furnishes a more delicate criterion of icterus in the dog than does that of the blood. The conditions are very different in man. In man, owing to the high renal threshold for bilirubin a considerable accumulation of pigment takes place in the blood before any escapes into the urine, and consequently one must reckon from a base-line of normal pigment retention in work upon defective bile elimination. This complicating factor does not exist in the dog and monkey. Such a renal threshold as is present in these animals—and *a priori* one would be expected on grounds of biological relationship—has so slight an elevation as to produce no evidence in the blood of its presence.

Method.

The animals were kept in metabolism cages and the 24 hour urines were examined for a number of days prior to operation, as regards their general character and the presence of bile salts and pigments. The monkeys were males, but most of the dogs chosen were females and with one exception (No. 10) none was taken which

[16] van der Bergh, A. A. H., and Snapper, J., *Berl. klin. Woch.*, 1914, li, 1109.

showed spontaneous icterus, so called. Throughout the work the routine tests were carried out upon cage urines, but positive findings were controlled by catheterization. At more or less frequent intervals, as occasion warranted, the blood was examined for bilirubin. The monkeys were fed on bananas, the dogs on bread and meat.

The biliary obstruction was induced by ligating and, where possible, cutting various branches of the hepatic duct under ether anesthesia. Asepsis was maintained, and the wound in the abdominal wall closed in three layers. When the free portion of the duct to be obstructed was too short to be doubly tied and cut, several stout ligatures were laid upon it, a procedure which in the dog generally served to close the duct throughout the term of experiment. Indeed, we have been but little troubled with the restoration of the duct channels by the cutting through of ligatures, possibly because of the large caliber of the silk used. In monkeys, on the other hand, even coarse ligatures were found to work through the duct walls within 10 days to 2 weeks, and either the continuity of the channel was reestablished, or a leak from it led to death. Needless to say, at every autopsy a careful study was made of the final results of operation and cultures were taken from the liver and the stasis bile. The animals were killed with chloroform. Instances in which infection existed were ruled from consideration.

Arrangement of the Dog and Monkey Livers.

The main liver mass of the dog consists of the left lateral and central and right central lobes with the ill defined quadrate lobe as an essential part of the last which also bears the gall bladder on its under surface.[17] There is another mass, separate both by contour and tissue cleavage, the lateral mass as we shall term it, which lies just above and to the right of the pylorus and is made up of the right lateral and caudate lobes. The main liver contains on the average about six-tenths of the parenchyma, the lateral mass about three-tenths. The remaining one-tenth consists of the papillary, or Spigelian, lobe placed below the main liver and separated from the lateral mass by the gastrohepatic omentum, but connected with both by tissue bridges. The course of the bile ducts falls in roughly with the anatomical arrangement of the tissue which they drain. A large branch coming from the left lateral and central lobes joins another from the right central and caudate lobes,—into which, by the way, the cystic duct opens,—and thus there is formed a large main channel into which the small papillary branch empties, and, lower down, within a few centimeters of the intestine, the considerable duct from the lobe mass. Frequent marked variations from this typical arrangement are encountered. Thus, for example, the branch from the lobe mass may course toward the main liver to join that from the right central and caudate lobes. Or the branch which appears to spring from the whole left central and

[17] Bradley, O. C., A guide to the dissection of the dog, London, New York, Bombay, and Calcutta, 1912.

TABLE II.

Weight of the Lobes of the Normal Dog Liver, Expressed in Percentages of the Total Organ.

Dog No.	Body weight.	Sex.	Liver weight.	Papillary lobe.	Lateral mass.		Entire lateral mass.	Main liver.			Entire main liver.
					Right lateral.	Caudate.		Left lateral.	Left central.	Right central.	
	kg.		*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	10	M.	315.2	5.8			29.0				65.2
2			181.5	7.3	19.5	9.8	29.3	25.4	11.5	26.5	63.4
3	7¾	M.	226.0	5.1	14.8	8.7	23.5	32.9	16.4	22.1	71.4
4	11	F.	330.5	6.1	16.8	14.8	31.6	24.5	13.6	24.2	62.3
5	15	M.	545.0	4.6	22.8	13.2	36.0	27.1	12.1	20.2	59.4
6	11		305.5	5.4	12.4	8.6	21.0	30.1	15.8	27.7	73.6
7	7	F.	212.5	4.7			34.0			19.1	61.3
8	11¾	M.	505.0				40.0				60.0
9	8	"	279.0				27.0				73.0
10	9¼	F.	288.0				22.7				77.3
11	11	M.	337.0	6.2			23.4			28.4	70.4
12	11¼	"	423.0				27.1				72.9
13	8	"	253.5				21.0	37.0			79.0
14	9	F.	333.5				25.8	27.2			74.2
15	7	M.	242.0				31.0				69.0
16	7	F.	335.0				20.0			21.8	80.0
17	8	M.	359.0	5.0			33.2				61.8
18	10¼	F.	359.5	4.6			32.0				63.4
19	9	"	351.0	6.0			27.1	25.0			66.9
20	8¾	"	335.0	4.9			25.3	27.7			69.8
21	7	"	231.5	4.1			21.6	28.5	18.6	27.2	74.3
22	12¾	M.	472.0	4.9			32.6	25.8			62.5
23	6	F.	285.0	4.6			36.8	24.5			58.6
24	8	"	252.5	3.9	21.6	14.5	36.1	27.7	11.7	20.6	60.0
25	6½	"	235.7	4.4	19.5	9.5	29.0	29.7	11.0	25.9	66.6

26	6½	F.	193.0	5.2	18.1	12.5	30.6	26.4	11.9	25.9	64.2
27	7¾	M.	261.5	4.6	22.8	14.7	37.5	26.8	11.1	20.0	57.9
28	8½	"	311.0	5.8	15.4	12.2	27.6	25.0	9.5	32.1	66.6
29	4½	F.	138.5	5.1	15.8	13.8	29.6	25.9	13.1	26.3	65.3
Average				5.1 (21 cases).	18.1 (11 cases).	12.0 (11 cases).	29.0 (29 cases).	27.6 (18 cases).	13.0 (12 cases).	24.5 (15 cases).	67.3 (29 cases).
Variation				3.9–7.3	12.4–22.8	8.6–16.9	20.0–40.0	24.5–37.0	9.5–18.6	19.1–32.1	57.9–80.0

lateral lobes may in reality drain but a small portion of these, their bile emptying for the most part into the just mentioned duct from the right central and caudate lobes. Large vasa aberrantia are frequent. They may be patent and filled with bile for several centimeters above their junction with the common duct, but stop short of the liver tissue or cease thereabouts to be patulous. A mistaken dependence upon such vessels for bile elimination has in some of our experiments led to the development of total obstruction when a partial one had been projected.

The liver lobes are in general defined by a cleavage of the parenchyma nearly to the hilum of the organ; and when there is but one duct from a lobe or group of lobes its obstruction leads to stasis throughout the tributary region. The proportion of the total liver affected can be readily determined under such circumstances. Potentially, at least, the case is different when there are two ducts or more to a lobe and one is left open. Under such circumstances relief may perhaps come to the area in stasis through newly opened channels into the unobstructed neighboring tissue. For it is well known that the bile canaliculi anastomose freely within the lobules. That no relief comes of a magnitude meriting consideration will be shown further on. The main difficulty lies in determining the exact amount of tissue in stasis.

Table II gives the weights of the liver lobes of twenty-nine normal dogs, expressed in percentages of the organ. In all cases the liver was removed before the blood had clotted. It will be seen that there is a rather large individual variation in the tissue distribution.

The monkey liver is divided into five lobes, much as in the rabbit, and these are grouped into a main liver and a lateral mass, which are connected at the base by a broad tissue bridge, as are the individual lobes also. A single short duct comes from each of the masses mentioned, and these unite to form an hepatic duct into which the cystic duct enters lower down, as in human beings. None of the ducts from the individual lobes save that from the caudate is accessible to ligation. This circumstance like that of the early cutting through of the ligatures has much hampered our observations.

"Physiological Jaundice."

Investigators upon icterus in the dog agree that it is frequently encountered in mild form in animals that appear normal. Naunyn[18] showed, as far back as 1869, that fasting for 24 hours regularly leads in most dogs to the appearance of bile pigment and salts in the urine. The icterus is not dependent on increased concentration of the urine, though it is made more evident thereby. A bilirubinuria from fasting was a frequent complicating factor in our early experiments, occurring

[18] Naunyn, B., *Arch. Anat., Physiol. u. wissensch. Med.*, 1869, 579.

regularly in the Sunday to Monday 24 hour specimen of unoperated animals as a result of the small ration of the day first mentioned. When a full diet was provided on Sundays intercurrent icterus became rare and often was traceable to disease. It was never found as the result of anesthesia or of the trauma of operation. In monkeys a fasting icterus was not noted, although it is said to develop occasionally in human beings.[3]

In view of these facts it seemed wise to keep several normal animals under observation with the operated ones as a control to intercurrent manifestations, and this was accordingly done.

Results of Total Obstruction in the Dog.

In sixteen dogs total obstruction was produced, sometimes by ligating and severing the common duct, but more frequently by cutting its large tributaries after their individual ligation. The tissue icterus that followed was never as pronounced as in human beings under similar conditions but in all cases was easily recognizable, while, in all, bile pigment became abundant in the blood and urine.[19] The tissue icterus was first visible on the 5th to 10th day, as yellowed scleras. The results in four of the sixteen dogs which were followed with special care have been tabulated (Table III).

The first sign of biliary obstruction was the appearance, as a rule, of pigment in the urine during the second or third 24 hours after operation but sometimes earlier. The delay is due, as Affanassiew showed,[20] to bile accumulation in the gall bladder and distended ducts: when he filled these reservoirs with wax at the time of operation icterus developed very much more promptly. Bilirubinemia was usually noted within 24 hours after bilirubinuria, and cholates were recognizable in the urine on the 3rd or 4th day. At autopsy of the dogs, after 9 to 46 days of obstruction, a general tissue jaundice was regularly found, the liver being especially affected.

[19] We have since autopsied an animal in which after 9 days of total obstruction no tissue icterus was discoverable. Urine and plasma were markedly tinted with bilirubin. There is little doubt that tissue pigmentation would have occurred in a day or so more.

[20] Affanassiew, M., *Z. klin. Med.*, 1883, vi, 281.

TABLE III.

Results of Total and Partial Obstruction in Dogs.

Dog No.	Sex.	Findings prior to operation.	Observed after operation for	Weight of animal.		Liver portion and per cent obstructed.	Postoperative findings.		Remarks.
				Initial.	Final.		Bilirubinemia.	Urine.	
			days	*kg.*	*kg.*				
1	F.	Negative for 9 days.	26	10¾	11	Total obstruction.	Positive on 3rd day and after.	Pigment on and after 2nd day; salts on 3rd day and thereafter.	Scleral jaundice appeared on 8th day.
2	"	Negative for 10 days.	15	9½	9½	" "	Positive on 2nd day and after.	Pigment on and after 2nd day; salts on 5th day and thereafter.	Scleral jaundice appeared on 5th day.
3	"	Negative for 7 days.	20	3¾	2½	" "	Positive on 4th day and after.	Pigment and salts positive on 3rd day and after.	Scleral jaundice appeared on 8th day.
4	"	Negative for 4 days.	13	8¾	8½	" "	Positive on 3rd day and after.	Pigment and salts on 3rd day and after.	Scleral jaundice appeared on 9th day.
5	"	Negative for 3 days.	26	10¼	10¾	All except lateral mass; 71 per cent.	None.	Negative.	
6	"	Negative for 4 days.	10	9½	9½	" "	"	" save for salts on 3rd day.	
7	"	Negative for 7 days.	37	7½	6¼	" "	"	Negative.	
8	"	Slight bilirubinuria of unknown cause during 5 days.	36	7¼	8¼	" "	"	Pigment and salts faintly positive on 2nd and 3rd days; salts positive on 16th, 20th, and 21st days.	Unoperated control animals showed pigment and salts likewise.

9	F.	Negative for 6 days.	23	5¾	5¼	All except left lateral lobe; 72.4 per cent.	None.	Pigment dubiously positive on two occasions.	Unoperated control animals showed pigment and salts likewise.
10	M.	Negative for 7 days.	34	17	16	" "	"	Pigment occasionally throughout.	
11	"	Negative for 6 days.	36	14¾	16¼	All except right lateral lobe; 82 per cent.	"	Salts from 7th to 14th days; thereafter, like pigment, negative.	Pigment once dubiously positive in urine.
12	F.	Negative for 7 days.	45	15¾	14½	" "	"	Salts from 8th to 37th days; pigment on 4th, 9th, and 42nd days.	
13	"	Negative for 7 days.	15	9½	9½	All except papillary lobe; 95 per cent.	Positive on 11th day and after.	Pigment on 2nd, 3rd, and 4th days; salts and pigment on 11th day and thereafter.	
14	"	Negative for 7 days.	15	6¾		" "	Positive on 2nd day and after.	Pigment on 2nd day and after; salts on 1st day and after.	
15	F.	Negative for 8 days.	28			Control animal.	None.	Pigment on 18th and 20th days; pigment and salts on 22nd, 24th, and 25th days.	
16	"	Negative for 4 days.	33			" "	"	Negative throughout.	
17	"	Negative for 5 days.	32			" "	"	Pigment on 11th, 12th, 13th, and 14th days; salts on 14th day.	
18	"	Negative for 5 days.	30			" "	"	Pigment and salts on 6th day and pigment on 19th day.	Pigment once dubiously positive in urine.

Results of Partial Obstruction in the Dog.

Partial obstruction was produced in ten dogs. Bile accumulation within these animals would presumably take place more slowly than on total obstruction, owing to the smaller amount of tissue in stasis and to the eliminative activities of the unobstructed portion. For these reasons no animal was considered to have given negative results as regards icterus until at least 10 days after operation and most were kept under observation for several weeks. As we shall point out further on, the unobstructed liver portion undergoes some hypertrophy within 10 days, while that in stasis atrophies, and thus the tendency to bile retention must soon be counteracted by lessened secretion in the region of stasis combined with increased facilities for elimination.

In four dogs approximately seven-tenths of the liver parenchyma was placed in stasis, in two others about three-fourths, in two, four-fifths, and in two about nineteen-twentieths. Table III summarizes the findings. Tissue icterus is not recorded because it was never observed, even when the eliminative burden had been thrown abruptly upon a mere twentieth of the liver.

In the four animals in which the duct from the lateral mass, draining on the average 29 per cent of the whole organ, was alone left open, bile pigment and salts were never demonstrable in blood or urine save on days when they were also present in control animals from the same intercurrent cause, namely, fasting. This was the case too when the free duct was that from the left lateral lobe containing 27.6 per cent of the tissue. A slightly greater degree of obstruction, produced by tying and cutting all the ducts except that to the right lateral lobe, or 18 per cent of the liver, resulted in the appearance of bile salts in the urine during the 2nd week after operation, occasionally accompanied in one animal by bile pigment as shown with the Gmelin test. The amount of pigment was always too slight to be quantitated, and bilirubinemia was not observed. Finally, when all the ducts were closed except that from the little papillary lobe, which holds from 3.8 to 7.3 per cent of the liver tissue, or on the average 5.1 per cent, pigment and cholates did indeed appear regularly in both blood and urine, and almost as rapidly as when total obstruction had been

produced. In the two instances studied a sufficient bile elimination took place, however, to prevent tissue icterus during the 15 days of observation, and in view of the papillary hypertrophy found at the end of this period there is but slight reason to suppose that a greater retention would have occurred later.

The four normal dogs that shared the general conditions of the operated animals and were followed in the same way yielded findings that were several times of great value, disclosing that bilirubinuria and choluria noted in the operated animals were "physiological," from food deprivation. In this connection it seemed of interest to determine whether animals with seven-tenths of the liver in bile stasis would show fasting icterus more readily than normal controls. Accordingly during a period of 6 days several animals of each sort were fed only a thin bouillon. This they took in quantity with result that the urinary output remained large. The experiment will be set forth in detail in a later paper on the physiology of jaundice. Here we shall merely state that while icterus appeared in most of the animals, it developed no sooner and was no more marked in the operated individuals than in the controls.

Liver Adaptation.

In dogs with total biliary obstruction jaundice of the liver parenchyma is outspoken after a few days. By contrast, when obstruction is partial, even when it affects nineteen-twentieths of the organ, an hepatic icterus is not observed. From this it is evident that vicarious bile elimination becomes effective very close to the source, so to speak, a fact which is not surprising when one considers that the hepatic cells lie in the midst of copious blood and lymph streams, which, if kept free of bile, should keep them free also.

Long continued obstruction to the ducts from a part of the canine liver results in noteworthy changes in the whole organ, just as in the rabbit[21] and in man.[22] The portion in stasis gradually becomes sclerotic by an interlobular proliferation of the connective tissue, and the parenchymal cells undergo a gradual simple atrophy, and may

[21] Nasse, *Verhandl. deutsch. Ges. Chir.*, 1894, xxiii, pt. 2, 525.

[22] Carnot, P., and Harvier, P., *Arch. méd. exp. et anat. path.*, 1907, xix, 76.

TABLE IV.

Results of Total and Partial Obstruction in Monkeys.

Monkey No.	Previous findings.	Observed after operation for	Weight of animal.		Liver portion and per cent obstructed.	Postoperative findings.		Remarks.
			Initial.	Final.		Bilirubinemia.	Bilirubinuria.	
		days	*gm.*	*gm.*				
1	Negative for 13 days.	7	1,900	1,900	Total obstruction.	Positive on 1st day and after.	Positive on 1st day and after.	Tissue icterus on 4th day and after.
2	Negative for 1 day.	8	2,375	2,300	" "	Positive on 1st day and after.	Positive on 1st day and after.	Tissue icterus on 3rd day and after.
3	Negative for 1 day.	11	2,300	2,375	" "	Positive on 2nd day and after.	Positive on 2nd day and after.	Tissue icterus on 3rd day and after.
4	Negative for 1 day.	12	3,100	2,900	" "	Positive on 5th day and after.	Positive on 5th day and after.	At operation a pathological dilatation of the bile passages was noted. Tissue icterus on 8th day and after.
5	Negative for 5 days.	12	2,300	2,325	All except lateral mass and papillary lobe; 75 per cent.	None.	None.	Duct continuity restored toward end of experiment.
6	Negative for 21 days.	11	1,975	2,025	All except lateral mass; 80 per cent.	"	"	Questionable restoration of duct's continuity toward end of experiment.
7	Negative for 5 days.	11	2,650	3,225	" "	"	"	Obstruction maintained.

ultimately disappear. Meanwhile, the tissue with duct unobstructed gradually hypertrophies. The underlying causes for these alterations have been analyzed in a previous paper from this laboratory.[1] In the dog they take place far more slowly than in the rabbit. A parenchymal shift is usually not discernible in less than 10 days, but after a month may be very marked. At this time the mass in stasis may be much shrunken, with finely hobnailed surface. Needless to say, instances free of infection are here alone referred to. The region with obstructed bile channels is now sharply demarcated from the adjacent hypertrophic tissue, and has been found to correspond closely in extent with the ramifications of these channels, thus proving that during stasis no important connections open between the blocked ducts and the neighboring free ones.

The shifting of tissue caused by local obstruction renders it impossible to determine exactly from the weights of the liver portions at autopsy how much of the parenchyma was originally placed in stasis. But the anatomical relations and a knowledge of the normal proportions of the liver lobes, such as Table II affords, enable one to reach an approximate conclusion on this point.

Results of Biliary Obstruction in the Monkey.

Seven monkeys were used (Table IV). They yielded results essentially similar to those in the dog. As in this animal, total obstruction was well tolerated, but bilirubinuria appeared more rapidly, developing in two out of four instances within the first 24 hours after operation. What would seem to be an interesting illustration of delay in its appearance owing to accumulation of bile in the ducts and gall bladder is afforded by Monkey 4. At operation a note was made that the bile passages of this animal, though undistended, were about three times the usual diameter. Despite the production of total obstruction, pigment failed to appear in urine or blood until the 5th day and tissue icterus was not seen until the 8th day. At autopsy all of the bile passages were enormously distended.

The plasma of monkeys subsisting on a banana diet is colorless and the urine nearly so. Bilirubinemia appears at approximately the

same time as bilirubinuria, on the 1st or 2nd day of total obstruction, and tissue icterus follows on the 3rd or 4th day. Cholates were never demonstrable in the urine by Hay's method. Further work would seem desirable on this point.

In three monkeys 75 to 80 per cent of the liver was placed in stasis. Owing to the rapidity with which ligatures cut through the ducts it was deemed best to terminate the experiments 11 and 12 days after operation. Even then in one instance there had been partial restoration of the duct continuity with some escape of bile into the intestine, though the well defined anatomical changes in the liver indicated that this was recent. At no time did the blood or urine of any of the animals contain bilirubin. That this would have continued to be the case is shown by the autopsy findings. For the livers, even in so brief a time, had undergone marked alterations in the direction of a functional readjustment. The unobstructed tissue was notably hypertrophied, and that in stasis shrunken and sclerotic. The changes had taken place almost as rapidly as in the rabbit, far more so than in the dog.

DISCUSSION.

The ability of a small portion of the liver to function for the whole as regards bile elimination when there is local obstruction depends without doubt upon several factors. That the parenchymal cells can rapidly excrete bile pigment and salts coming to them in large amount on the blood stream is proven by the promptness with which these substances pass into the bile when thrown into the circulation for experimental purposes.[23] Bile constituents formed by the tissue in stasis, and carried away from it by the blood and lymph, will of course be treated similarly. But the conditions of stasis themselves tend to lessen the amount of bile formed. For the obstructed ducts, dilating under pressure, interfere with the local portal flow[24] and divert a portion of the blood and with it the functional activities, to the more normal hepatic regions. There follows in due course a pa-

[23] Wertheimer, E., *Arch. physiol. norm. et path.*, 1892, iv, series 5, 577. Stadelmann, E., *Deutsch. med. Woch.*, 1896, xxii, 785.

[24] Betz, W., *Sitzungsber. k. Akad. Wissensch. Math.-naturw. Cl., Wien.*, 1862, xlvi, 238.

renchymal shifting from the region in stasis,[1] which, as time passes, tends to become complete. It is possible, furthermore, that stasis directly influences the liver cells to form less bile, but this cannot be profitably discussed.

Attempts should be made to determine in human beings the margin of safety in bile elimination. Quantitative studies on the rate of disappearance of jaundice after the surgical relief of total biliary obstruction should provide interesting figures on the ability of the liver to excrete pigment in excess. Injections post mortem into the bile ducts to determine the degree of obstruction in congenitally cystic livers and livers with widespread carcinomatosis ought, in connection with the clinical findings, to yield data of value. Perhaps the most direct evidence, though, is to be had from cases of local obstruction by intrahepatic calculi.

Beer,[25] who described many instances of stones within the liver, remarks on the fact that even when numerous and widely distributed they often cause no symptoms during life. Lewisohn[26] tells of finding at operation a liver studded with fibrous nodules in each of which was one or more gall stones. Yet the patient had for a long time been in good health and unjaundiced. Carnot and Harvier[22] report the complete atrophy of a liver lobe as result of an intrahepatic calculus occurring in a patient who was never jaundiced. Such instances, as well as those more frequent ones of disseminated hepatic carcinomatosis without jaundice, which come under clinical observation, give good reason for the belief that the human liver possesses a margin of safety in bile elimination not inferior to that of the dog and monkey.

There is a point of immediate practical import in the demonstration that the biliary obstruction required to produce jaundice is one affecting the greater portion of the liver. Jaundice is not infrequently seen in association with abscesses and other less discrete inflammatory changes occupying but a small portion of the hepatic tissue. The inference from such observations has been that the jaundice results from local bile resorption. But in view of our

[25] Beer, E., *Med. News*, 1904, lxxxv, 202.
[26] Lewisohn, R., *Ann. Surg.*, 1916, lxiii, 535.

findings this would seem highly unlikely. Rather should one think in such instances of a general injury either to the liver parenchyma or ducts, or else to the blood corpuscles.

SUMMARY.

The bile ducts from three-quarters of the liver substance in dogs and monkeys can be obstructed without any clinical evidence developing of pigment or cholate accumulation in the organism. And in the dog nineteen-twentieths of the liver substance can be placed in stasis without the occurrence of tissue icterus such as regularly follows total obstruction in this animal. There is no reason to suppose that this will not be found true in the monkey as well. Always a local obstruction results sooner or later in atrophy of the affected tissue with compensatory hypertrophy elsewhere. Thus as time passes the derangement of function produced by the sudden stasis is progressively lessened.

The plasma of the dog and monkey, unlike that of man, is normally free from bilirubin, and this pigment so readily escapes from the blood into the urine that bilirubinuria is often to be found in the dog in the absence of bilirubinemia, while the latter is never met with alone in either animal. It follows that in both species the renal threshold for bilirubin is much lower than in man,—if indeed one can be said to exist at all.

The amount of biliary obstruction required to produce jaundice in human beings is probably as great as in the experimental animals with which we have dealt. The clinical jaundice encountered in association with local liver lesions should be viewed not as the result of local bile resorption, but as due to a general injury to the hepatic parenchyma or ducts, or to blood destruction.

TUBERCULIN HYPERSENSITIVENESS IN NON-TUBERCULOUS GUINEA PIGS INDUCED BY INJECTIONS OF BACILLUS-FREE FILTRATES.

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INTRODUCTION.

The hypersensitiveness of the tissues of a tuberculous animal to tuberculin provides a means for the accurate determination of infection with *Bacillus tuberculosis* in man and domestic animals and it has led to much valuable experimental work to explain its mechanism. Koch's observation that tuberculous animals resist more or less successfully attempts at reinfection has been repeatedly confirmed, and since the introduction by Römer and Joseph[1] of the intracutaneous tuberculin test which is applicable to guinea pigs, evidence has been accumulating to show that there is a parallelism between intensity of the local response to tuberculin and resistance to reinfection. Baldwin[2] found that small injections of killed tubercle bacilli and various extracts containing the protein of the bacillus sensitize to tuberculoprotein so that anaphylactic shock may be elicited, but that such sensitized animals give no cutaneous response to tuberculin. Living virulent tubercle bacilli inclosed in Berkefeld filters and placed in the peritoneal cavity of guinea pigs did not lead to the development of a tuberculin reaction in the animals. In other experiments he found that repeated small injections of charcoal saturated with a filtrate of tubercle bacillus extracts, with a benzene

[1] Römer, P. H., and Joseph, K., *Berl. klin. Woch.*, 1909, v, 1300.

[2] Baldwin, E. R., *Tr. Nat. Assn. Study and Prevent. Tuberc.*, 1911, vii, 351.

extract containing tubercle bacillus wax, or with whole blood or sera from tuberculous animals resulted in no response to tuberculin. A number of these experiments have been repeated and extended by Krause[3] and by others. Krause summarizes the results in the statement that: "There is no cutaneous hypersensitiveness without a focus (tubercle)." It was found by the author[4] in the course of some experiments to determine the effect of non-pathogenic acid-fast bacilli on the blood leucocytes that injections into guinea pigs of large and small doses of living cultures of non-virulent tubercle bacilli (saprophytic) were not followed by a development of the tuberculin reaction.[5]

If the tuberculin reaction is dependent upon a hypersensitiveness to a foreign protein, then the failure of living tubercle bacilli (saprophytic) which have lost their virulence to bring about a tuberculin response means presumably that the protein of these bacilli has undergone an essential change. That killed cultures of virulent tubercle bacilli and various derivatives of these may not contain the identical protein present in living virulent ones and do not therefore sensitize to the virulent bacilli or their products seems not unlikely in view of the vigorous means required to kill organisms of the acid-fast group. No more definite statement in regard to the very general characters of protein sensitization is possible than that subsequent to the sensitizing dose some soluble substance passes out from the blood or focal area of tissue receiving the injection to all the cells and tissues of the organism with the result that the cells and tissues acquire after a certain time a new property which renders them susceptible to toxic injury when brought into contact with the same protein. In tuberculosis whether the substance passing out from the focus of infection, the tubercle, to give the tuberculin reaction is the protein of the

[3] Krause, A. K., *J. Med. Research*, 1916–17, xxxv, 21; *Am. Rev. Tuberc.*, 1919, iii, 153.

[4] McJunkin, F. A., *J. Med. Research*, 1921, xlii, 201.

[5] One of the so called saprophytic cultures was received from the Hygienic Laboratory at the University of Michigan through the courtesy of F. G. Novy, and the other from the University of Cincinnati through the courtesy of W. B. Wherry. Both grow rapidly on simple media and neither produces tuberculosis in guinea pigs.

virulent tubercle bacillus, a split-product of this protein, or some substance resulting from the reaction of the tissue cells in the tubercle is uncertain. However this may be, it seems likely that when virulent tubercle bacilli are injected into the peritoneal cavity of tuberculous guinea pigs a certain number of bacilli are broken up into granules and dissolved in much the same way as some of them are in tubercles, and that any further action on the protein of the virulent bacilli which may follow their disintegration and solution would be of the same general nature. Kraus and Hofer[6] state that such solution of tubercle bacilli follows their injection into the peritoneal cavity of tuberculous guinea pigs; probably the so called Much granules found in "cold" abscesses are evidence of the breaking up of tubercle bacilli.

Technique of the Intracutaneous Tuberculin Test.

In a small syringe provided with a 27 gauge $\frac{3}{4}$ inch needle 0.02 cc. of tuberculin[7] is diluted up to 0.1 cc. with saline solution. The needle is inserted, preferably with the eye outward, into the shaved skin of the lightest colored portion of the abdomen and sufficient diluted tuberculin injected to raise a 1 cm. bleb. This amount, which is less than half that used by Römer and Joseph and by others tends to give a reaction more definitely localized than larger amounts. When introduced properly the point of the needle is distinctly seen and the liquid is forced out against considerable resistance, while if the needle is inserted into the subcutaneous tissue the liquid is easily forced out and the bleb which is formed does not remain long. The technique requires care in animals weighing less than 400 gm. In testing animals it is always advisable to inject the tuberculin into one or more normal guinea pigs of about the same age as controls. In the negative reactions the puncture is usually visible at the end of 24 hours but there is no redness or induration. In the lightest reactions ("lightly positive") recorded as positive there is a 1 cm. area of redness with slight swelling at the end of 24 hours. In the reactions recorded as "moderately strong" there is a 1 or 2 cm. area of local-

[6] Kraus, R., and Hofer, G., *Deutsch. med. Woch.*, 1912, xxxviii, 1227.

[7] The tuberculin (O. T.) was obtained from H. K. Mulford Company.

ized redness and distinct induration at the end of the 24 hours, while in the "strongly positive" reactions necrosis and sloughing take place. In the reactions characterized by necrosis a central opacity appears in 24 hours, and by the end of 48 hours hemorrhage into a necrotic center frequently occurs and an open ulcer follows. Lightly positive reactions have not been relied upon to any extent, but they appear to be trustworthy if careful controls in sufficient numbers are employed. Moderately strong responses also should be carefully controlled by simultaneous tests on normal guinea pigs. A guinea pig that has received an injection of virulent bacilli becomes positive in about 2 weeks.

EXPERIMENTAL.

The experiments consist essentially of the treatment of normal guinea pigs with a bacillus-free filtrate obtained from the peritoneal cavity and organs of tuberculous guinea pigs 6 to 24 hours after intraperitoneal injections of large amounts of heavy suspensions of living virulent tubercle bacilli.

In the first experiment two guinea pigs were injected with a bacillus-free filtrate, No. 23. To prepare this filtrate a 320 gm. tuberculous guinea pig which had been inoculated intraperitoneally 37 days before with Culture H37 and had shown a strongly positive reaction to tuberculin was injected intraperitoneally at 2 p.m. on Dec. 13, 1920, with the growth from a 6 week flask culture of Culture H37 in 20 cc. of glycerol bouillon. After the removal of all except about 5 cc. of the bouillon the growth was ground in a mortar and suspended in 16 cc. of 2 per cent gelatin. 5 p.m. Animal alive. Dec. 14, 9 a.m. Found dead. The peritoneal cavity was opened and 20 cc. of clear and somewhat viscid exudate were removed and the undiluted liquid was passed through a $2\frac{1}{2}$ inch Berkefeld filter (N). The omentum was gathered in a caseous mass but the other organs showed no extensive lesions. A film of the unfiltered peritoneal exudate showed a few neutrophils and a few mononuclear cells. An occasional leucocyte of both varieties contained a few tubercle bacilli. There were present a few clumps of extracellular acid-fast granules and an occasional intracellular one, and although they appeared to be partially dissolved bacilli it is not possible to identify them positively as such. About 10 cc. of clear filtrate were obtained. The culture, No. H37, is a human type which grows well on Dorset's egg medium and on glycerol bouillon, develops a positive tuberculin reaction in guinea pigs in the usual time, and causes their death in 6 weeks to a number of months according to the size of the dose and the age of the animal.[8]

[8] The culture was obtained from the laboratory of A. I. Kendall of Northwestern University Medical School where it is known as Culture H37.

Guinea Pig 39.—Weight 300 gm. Received a subcutaneous injection in the right groin of 1 cc. of No. 23 filtrate on Dec. 14, 1920, immediately after the filtration had been completed, 1 cc. on Dec. 17, and 2 cc. on Dec. 21. At this time a tuberculin test was made and the following day there was redness at the site of injection, but owing to a slight abrasion of the skin due to careless shaving this was not recorded as positive. Dec. 22. The test was repeated and gave a positive reaction the following day. Dec. 24. Test was recorded as moderately strong. Dec. 27. Tuberculin was again administered. Dec. 28 and 29. Test recorded as strongly positive. Dec. 30. A 1 cm. ulcer was present at the site of the last tuberculin injection. Dec. 21. The filtrate injections were discontinued. Jan. 6, 10, 12, and 19, 1921. The tuberculin test was moderately strong or lightly positive. Jan. 31. The test was negative. On Feb. 1, 49 days after the first filtrate injection, the animal, which then weighed 520 gm., was chloroformed.

Autopsy.—Nothing suggesting a tuberculous process was found. One or two translucent lymph nodes measuring 2 or 3 mm. were found in the superficial part of the groin fat. These as well as some of the deeper fibrous tissue were smeared on slides and stained by the Ziehl-Neelsen method; no acid-fast bacilli were found. No tubercles were visible to the naked eye in any of the organs and histologic examination of the omentum, spleen, liver, and lungs was negative.

Guinea Pig 41.—Weight 440 gm. Dec. 24, 27, and 30, 1920, and Jan. 1, 1921. Given 1 cc. of Filtrate 23. Jan. 1. A tuberculin test was negative. Jan. 3. Tuberculin was again tried and on Jan. 4 and 5 there were slight reddening and slight induration. Jan. 6. A similar response was obtained. These reactions were recorded as lightly positive. Jan. 8, 10, 19, and 26, 1921. The tests were negative. On Dec. 24, when the first injection was given, the filtrate, which had stood in the ice box for 9 days, was somewhat clouded and showed some bacterial contamination. Feb. 1. Animal weighs 660 gm. It is to be kept for a number of months.

In the second experiment four guinea pigs were injected with filtrates; two received a filtrate prepared in much the same way as that used for the injection of Guinea Pig 39 while the other two received a filtrate which was obtained by crushing the tissues of the abdominal wall and certain of the organs. These filtrates were obtained from Guinea Pig 40 which weighed 670 gm. and had developed a strongly positive tuberculin reaction 7 days after a large groin inoculation with Culture H37. The suspension of tubercle bacilli for injection into the peritoneal cavity of this tuberculous guinea pig was prepared in the same way as that used in the first experiment to obtain Filtrate 23, except that 30 cc. of 2 per cent gelatin were used to suspend the bacilli and all except about 1 cc. of the bouillon was removed. After 24 hours the animal was alive but appeared very toxic. It was killed with chloroform and the 15 cc. of exudate obtained from the peritoneal cavity were diluted with 15 cc. of saline solution and passed through a Berkefeld filter. The heart, lungs, kidneys, a portion of the liver, and muscle

from the abdominal wall were ground in a mortar, mixed with 50 cc. of saline solution, and the liquid was passed through another filter.

Guinea Pig 46.—Weight 460 gm. Injected in the groin with 6 cc. of filtrate obtained from the peritoneal exudate on Dec. 30, 1920, 4 hours after the animal had been chloroformed and the filtration started. On Jan. 1, 1921, injected with 2 cc., Jan. 3 with 1 cc., and Jan. 5 with 3 cc. Jan. 6. The tuberculin test was lightly positive. Jan. 10, 12, and 19. Test moderately strong. Jan. 26. The test was lightly positive.

Guinea Pig 47.—Weight 210 gm. Received the same filtrate on the same days as No. 46, but the quantity given was somewhat less and averaged 2 cc. per dose. The responses to tuberculin were the same.

Guinea Pigs 48 and 49.—Weight 260 and 220 gm. respectively. Received groin injections of 2 cc. of filtrate obtained from the crushed organs on Dec. 30, 1920, and 1 cc. on Jan. 1, 3, and 5, 1921. There was no response to tuberculin tests until Jan. 10 when both animals gave moderately strong reactions. Jan. 12. The same responses were present. Jan. 19 and 26. The tests were lightly positive.

Autopsies.—Feb. 1. Guinea Pigs 47 (weight 340 gm.) and 48 (weight 360 gm.) were chloroformed and films were made and examined after staining by the Ziehl-Neelsen method. In both these animals just beneath the groin fat a small bit of injected tissue was present. This slight local reaction was thought to be due to tuberculin which had been injected Jan. 31 and indicates that the tissues at the site of the filtrate injections are more sensitive than the skin a short distance removed. This reddened tissue was smeared out on slides and stained. No tubercle bacilli were found in these films or in those made from the small lymph nodes. Histologic examination of the omentum, spleen, lungs, and liver showed nothing which suggested tubercles. Guinea Pigs 46 and 49 are to be kept for a number of months.

In the third experiment (Table I) twenty-one guinea pigs were injected in the left axilla with filtrate as follows: Jan. 17, 1921, 2 cc. of Filtrate 45 (Table II); Jan. 19, 5 cc. of Filtrate 51; Jan. 21, 5 cc. of Filtrate 11; Jan. 24, 4 cc. of Filtrate 43. Animals 62, 63, 74, and 75 were placed in the series as controls and received no filtrate injections. In order to secure a more concentrated filtrate such as the one used for Guinea Pig 39 in the first experiment exceptions were made in six guinea pigs, Nos. 54, 55, 56, 67, 68, and 69, which in addition to the filtrate injections noted above were given on Jan. 27 injections of 5 cc. of Filtrate 80; on Jan. 29, 5 cc. of Filtrate 12; and on Feb. 2, 3 cc. of Filtrate 81. There was no demonstrable general or local reaction in any of the animals until the day following the fourth injection, Jan. 25, when a very slight amount of induration appeared in the axillary tissue. Feb. 3. Nos. 54, 55, 56, 67, 68, and 69 were the only ones that showed induration in the axilla and in these the reaction consisted of a slight and somewhat indefinite thickening of the axillary tissue. When the results of the tuberculin test were seen on Jan. 28 it was thought that the

dilution of the filtrate used in the first four injections was too great to secure the response obtained in Guinea Pig 39 which received about half of the total amount of filtrate obtained from one animal.

The filtrates for the third experiment were prepared from guinea pigs tabulated in Table II. The suspensions were made from a 3 to 6 week growth of

TABLE I.

Guinea pig No.	Weight.	Dates of tuberculin tests and responses.							
		Jan. 27.	Jan. 28 and 29.	Jan. 31.	Feb. 1 and 2.	Feb. 8.	Feb. 9.		Feb. 10 and 11.
	gm.								
54	310	Test.	±*	Test.	+	Test.	±	Test.	++
55	310	"	±	"	+	"	+	"	++
56	310	"	−	"	+	"	+	"	++
57	400	"	+	"	+				
58	320	"	±	"	+				
59	320	"	±	"	+				
60	340	"	±	"	+				
61	360	"	+	"	+				
62	360	"	−	"	−				
63	420	"	−	"	−				
64	400	"	+	"	+				
65	380	"	+	"	+				
66	400	"	±	"	+				
67	400	"	−	"	+	Test.	+	Test.	++
68	400	"	−	"	+	"	+	"	++
69	410	"	−	"	+	"	+	"	++
70	400	"	−	"	+				
71	340	"	±	"	+			Test.	−
72	330	"	±	"	++				
73	360	"	±	"	+				
74	340	"	−	"	−				
75	330	"	−	"	−			Test.	−
76	340	"	−	"	+			"	+
77	340	"	±	"	+				
78	360	"	−	"	+				

* A lightly positive reaction is indicated by +, a moderately strong by ++, and a strongly positive by +++. Doubtful reactions are indicated by ±.

Culture H37 on about 20 cc. of glycerol broth contained in a 150 cc. Erlenmeyer flask. The growth was allowed to continue until a film spread over the entire surface of the bouillon; the contents of the flask were then placed in a mortar, much of the broth was removed, and the growth was ground for a few minutes. A variable amount of saline solution was used for making the final suspensions

TABLE II.

Guinea pig No.	Date.	Weight.	Treatment.	Tuberculin test.	Filtrate obtained.
	1920	*gm.*			
3	Sept. 30	425	Intraperitoneal inoculation with Culture H37.		Filtrate from incubated organs and bacillus suspension.
	Nov. 29	540	Heart's blood and organs removed aseptically.	+++	
11	Oct. 5	420	Intraperitoneal inoculation with Culture H37.		110 cc. of filtrate from peritoneal cavity and organs ground in mortar, which is about one-half the fluid used for dilution.
	1921				
	Jan. 20		20 cc. of suspension of Culture H37 injected intraperitoneally.	+++	
	" 21		Dead.		
	1920				
12	Oct. 6	410	Intraperitoneal inoculation with Culture H37.		30 cc. of filtrate from peritoneal cavity and organs, which is about one-half the fluid used for dilution.
	1921				
	Jan. 28		40 cc. of suspension of Culture H37 injected intraperitoneally.	+++	
	" 29		Dead.		
	1920				
23	Nov. 6	320	Intraperitoneal inoculation with Culture H37.		About 10 cc. of filtrate from undiluted peritoneal exudate.
	Dec. 13		16 cc. of suspension of Culture H37 injected intraperitoneally.	+++	
	" 14		Dead.		
29	" 1	580	Subcutaneous inoculation with Culture H37.		Filtrate from incubated organs and bacillus suspension.
	" 20		Heart's blood and organs removed aseptically.	+++	
40	" 22	670	Subcutaneous inoculation with Culture H37.		About 15 cc. of filtrate from peritoneal cavity and the same amount from the crushed organs; in each case this was about one-third the fluid used for dilution.
	" 29		30 cc. of suspension of Culture H37 injected intraperitoneally.	+++	
	" 30		Killed with chloroform 21 hrs. after injection.		

TABLE II—*Concluded.*

Guinea pig No.	Date.	Weight.	Treatment.	Tuberculin test.	Filtrate obtained.
	1920	*gm.*			
43	Dec. 28	640	Subcutaneous inoculation with Culture H37.		95 cc. of filtrate from peritoneal cavity and the organs, which is less than one-half the saline solution used for dilution.
	1921				
	Jan. 23		20 cc. of suspension of Culture H37 injected intraperitoneally.	++	
	" 24		Killed with ether 23 hrs. after injection.		
	1920				
45	Dec. 28	610	Subcutaneous inoculation with Culture H37.		54 cc. of filtrate from peritoneal cavity and the organs, which is less than one-half the saline solution used for dilution.
	1921				
	Jan. 16		30 cc. of suspension of Culture H37 injected intraperitoneally.	++	
	" 17		Killed with ether 21 hrs. after injection.		
51	" 5	720	Subcutaneous inoculation with dilute tuberculous sputum.		105 cc. of filtrate from peritoneal cavity and the organs, which is one-half the saline solution used for dilution.
	" 18		30 cc. of suspension of Culture H37 injected intraperitoneally.	++	
	" 19		Killed with ether 23 hrs. after injection.		
80	" 16	640	Subcutaneous inoculation with Culture H37.		30 cc. of filtrate from peritoneal cavity and the organs, which is less than one-half the saline solution used for dilution.
	" 26		10 cc. of suspension of Culture H37 injected intraperitoneally.	++	
	" 27		Killed with ether 23 hrs. after injection.		
81	" 18	600	Subcutaneous inoculation with Culture H37.		15 cc. of viscid cloudy fluid were mixed with 25 cc. of saline solution used to wash out the peritoneal cavity. 18 cc. of filtrate obtained.
	Feb. 1		15 cc. of suspension of Culture H37 injected intraperitoneally.	+	
	" 2		Etherized 22 hrs. after injection.		

which were injected into the peritoneal cavity by means of a large gauge needle. In preparing the filtrates for the first and second experiments a 2 per cent gelatin instead of saline solution was used for suspension. As shown in Table II guinea pigs with a strongly positive tuberculin reaction die between the 8th and 21st hours following injections of the suspensions, while those with a less intense reaction live for a greater length of time. The peritoneal exudate was always mucoid in character and sometimes strikingly so. The amount, which was variable, was greatest when a large amount of suspension was injected. With single filtration through a fine filter the filtrate obtained in 4 to 7 hours was not more and often considerably less than one-half the volume of the combined saline solution used for washing out the peritoneal cavity and extracting the ground pulp of the organs. Filtration was usually started about 10 a.m. and finished between 1 and 5 p.m. The injections of the animals with the filtrate were made at once. Cultures of the filtrates on agar slants remained sterile. That the tuberculin test was positive and then became negative or of a less intensity in Guinea Pigs 39, 41, 46, 47, 48, and 49 seems to be conclusive proof that tubercle bacilli did not pass the filters. Guinea Pigs 52, 16, and 26 (see text below) received filtrates from similar heavy suspensions of tubercle bacilli and remained unresponsive to tuberculin.

Guinea Pig 52.—Weight 290 gm. Received 15 cc. of Filtrate 44 between Jan. 6 and 11, 1921, and gave repeated negative or doubtful reactions to tuberculin. Filtrate 44 was obtained from a 680 gm. guinea pig on the 7th day after a groin inoculation with Culture H37 when the tuberculin test was lightly positive. The amount of filtrate obtained was about 75 cc., which was less than one-third the amount of saline solution used for dilution. Jan. 28. The test was still negative.

Filtrates obtained by filtering mixtures of the incubated organs of two tuberculous guinea pigs and heavy suspensions of Culture H37 were injected into Guinea Pigs 16 and 26 but a typical response to tuberculin was not obtained. Guinea Pig 16, which weighed 400 gm., received on Dec. 21 to 30, 1920, four injections of 2 cc. each of Filtrate 29. This filtrate had been obtained by removing aseptically and grinding in a mortar the spleen, kidney, and 6 cc. of heart's blood of a tuberculous guinea pig with a strongly positive tuberculin reaction, mixing the paste with 10 cc. of a heavy suspension of Culture H37, incubating over night at 37°C., diluting with 20 cc. of saline solution, and filtering. Guinea Pig 26, which weighed 600 gm., received on Dec. 1 to 27, 1920, ten injections of 2 cc. each of Filtrate 3. This filtrate had been obtained from a tuberculous guinea pig with a strongly positive test by the same method used for No. 29 except that a tuberculous focus in the omentum was ground up with the organs. A typical response to tuberculin was not obtained.

DISCUSSION.

A positive tuberculin test develops in about the same time after the filtrate injections as it does after inoculations with tubercle bacilli. The presumption is that the substance in the filtrate which leads to the tuberculin hypersensitiveness is identical with that present during the first few days in a tuberculous focus produced by a subcutaneous inoculation with living virulent tubercle bacilli and that it is a product of the action of the tissues of the tuberculous animal on the tubercle bacilli contained in the suspension introduced into the peritoneal cavity. There is some rather inconclusive microscopic proof that a disintegration and solution of some of the injected bacilli take place. In view of the many instances of the bacteriolysis of other bacteria under more or less analogous conditions this seems especially probable.

It has been shown by a number of investigators that guinea pigs hypersensitive to tuberculin are resistant to reinoculation. Experiments are now under way to determine whether the same is true of guinea pigs made sensitive to tuberculin by the injection of the filtrates. In order to induce an effective resistance to inoculation it seems reasonable to suppose that it will be necessary to establish by the filtrate injections a strongly positive tuberculin reaction. From the experiments it is evident that the substance in the filtrates which produced a hypersensitiveness to tuberculin when injected into normal guinea pigs varied quantitatively in the different tuberculous animals from which the filtrates were obtained. It appears that guinea pigs with a strongly positive tuberculin reaction as a result of peritoneal tuberculosis yield the most active filtrate.

SUMMARY AND CONCLUSIONS.

1. When a guinea pig with well developed peritoneal tuberculosis is injected intraperitoneally with about 20 cc. of a heavy suspension of a virulent tubercle bacillus (Culture H37) death occurs within 24 hours or the animal becomes extremely toxic. Such a peritoneal tuberculosis develops in about 1 month after 1 cc. of a very heavy suspension of Culture H37 has been introduced into the abdominal cavity. If the viscid fluid which is contained within the peritoneal

cavity is mixed with saline solution and passed through a Berkefeld filter a bacillus-free filtrate is obtained which induces in normal guinea pigs a certain degree of cutaneous hypersensitiveness to tuberculin.

2. The abdominal organs and the parietal peritoneum, to which masses of leucocytes and tubercle bacilli are adherent, when crushed and extracted with saline solution yield a filtrate which likewise induces a cutaneous hypersensitiveness.

3. The cutaneous hypersensitiveness does not appear before the 7th or 8th day after the filtrate injection and is therefore considered to be the result of an active sensitization of the animal.

STUDIES ON BACTERIAL NUTRITION.

I. Growth of Bacillus influenzæ in Hemoglobin-Free Media.

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Pfeiffer in 1892 first obtained growth of *B. influenzæ* by the use of agar slants, the surface of which was smeared with a few drops of human blood. Pfeiffer showed that this bacillus grew only in media containing blood or hemoglobin. The influenza bacillus was thus brought into the group of the hemoglobinophilic bacilli and has since always been considered an obligate hemophilic organism despite the fact that the literature shows that several investigators have been able to cultivate it in media free of blood or hemoglobin.

The first to show this fact was Cantani (1), who in 1901 obtained growth of *B. influenzæ* on ascites agar in symbiosis with other bacteria, such as gonococcus, diphtheria bacillus, and several large cocci. *B. influenzæ* grew in giant colonies in the vicinity of the other colonies. Cantani also obtained growth on the surface of agar to which had been added emulsions of bacteria killed by heat at 60°C. for 3 hours. Cantani excludes symbiosis and supposes that some factor in the bacterial cell induces the growth of *B. influenzæ* and that this factor is more easily liberated from the dead than from the living cell.

Neisser (2), however, demonstrated the growth of influenza bacilli in symbiosis with xerosis bacilli on plate cultures made from the inflamed conjunctivæ of a child. Impure colonies were observed which could be transferred through many generations on plain agar without the disappearance of *B. influenzæ*. The latter, however, failed to grow on this medium if the xerosis bacillus was not present.

Neisser also tried to cultivate *B. influenzæ* on media prepared by adding killed emulsions of the xerosis bacillus or the diphtheria bacillus to plain agar. A slight growth occurred for three or four generations, but the continued cultivation of *B. influenzæ* on this medium was impossible. He therefore considered the growth of *B. influenzæ* in mixed cultures as a symbiotic phenomenon.

Grassberger (3) observed that when *B. influenzæ* was grown on blood or hemoglobin agar plates in association with other bacteria, especially staphylococci, the colonies of influenza bacilli adjacent to the colonies of cocci were of unusually large size. He assumed that the cocci exerted some effect upon the blood medium that was beneficial for the growth of *B. influenzæ*.

Luerssen (4) confirmed the observations of Cantani by growing *B. influenzæ* on agar containing emulsions of dead staphylococci, *B. coli*, or *B. prodigiosus*. Growth of *B. influenzæ* did not occur in ordinary mixed cultures with these other organisms. The bacterial emulsions were sterilized by heating at 60°C. for 3 hours; if they were boiled the growth which occurred was not so good. Luerssen presumes that the bacteria contain a factor that exerts a stimulating effect upon the growth of *B. influenzæ*, and that this is destroyed at high temperatures. He found that this growth factor is contained in the bacterial cell, since the carefully washed cells are still active. Growth of *B. influenzæ* did not occur if the enriching emulsion of dead organisms was smeared on the surface of the agar; it only occurred if the emulsion was incorporated in the medium. Luerssen also observed that *B. influenzæ* grew sparsely in sterile filtrates of broth cultures of staphylococci, *B. diphtheriæ*, and *B. violaceus*.

Ghon and von Preyss (5) attempted to grow *B. influenzæ* in media containing no hemoglobin, but the results were negative and they concluded that the reason other investigators had been successful was that in making the inoculations small amounts of blood had been carried over. They likewise held the opinion that when *B. influenzæ* grows on plain agar, as occasionally happens, this agar contains traces of hemoglobin.

Recently Putnam and Gay (6) tried to confirm the experiments of the earlier investigators. They were unable, however, to obtain any growth of *B. influenzæ* on plain agar to which either killed or living cultures of *B. xerosis*, *B. diphtheriæ*, *B. coli*, or staphylococci had been added.

In spite of the observations concerning the growth of *Bacillus influenzæ* in media free of blood or hemoglobin, the opinion that this organism is hemoglobinophilic has not been altered, and for its cultivation media containing blood or hemoglobin in some form is always employed.

EXPERIMENTAL.

In a study on the growth of mucoid bacilli and the transformation of non-mucoid bacilli into mucoid ones (7) the writer undertook the cultivation of different microbes, *Bacillus paratyphosus* B and pneumococci, in broth containing mucus produced by mucoid bacilli such as Friedländer's bacillus, the ozenabacillus, and other similar organisms. An attempt was also made to cultivate *Bacillus influenzæ* in this medium. The development of this work led to the present studies on bacterial nutrition.

Experiment 1.—A culture of a mucoid Gram-negative bacillus (Friedländer's) was grown on plain agar. After 24 hours in the incubator the growth from each plate was suspended in a few drops of plain broth. 0.5 cc. of this emulsion was added to 5 cc. of plain broth, then heated for 1 hour at 60°C., tested for sterility, and stored in the ice box. The reaction of the broth, pH 7.8, was not altered by the addition of the bacterial emulsion. With the medium prepared in this manner, the following tests were made.

A tube of this medium was inoculated with influenza bacilli from a blood broth culture, the blood cells of which had completely settled to the bottom of the

TABLE I.

Growth of B. influenzæ in Plain Broth Added to Emulsions of Friedländer's Pneumonia Bacillus.

No. of transfers from original culture in emulsion broth.	Age of culture when transferred.	Growth of culture on blood agar after standing in incubator.									
		No. of days.									
		1	2	3	4	5	6	7	8	9	10
	hrs.										
Emulsion broth 1	48	++	++				0				0
	days										
" " 2	4				+						+
	hrs.										
" " 3	24	++							+		
" " 4	48	++			++			++			
" " 5	48	++			++						0
	days										
" " 6	5	++	++								0
	hrs.										
" " 7	24	++			++						
" " 8	48	++			++						
" " 9	Not transferred.	++									

tube. The following day 0.2 cc. of this subculture was transferred to another tube of emulsion broth. Simultaneously a loopful of the first culture was streaked on the surface of blood agar to demonstrate the growth of *B. influenzæ*. The last procedure was necessary since the emulsion broth in itself was too cloudy to show growth. From the first culture there were made eight successive transfers to emulsion broth, in all nine transfers from the original blood broth culture.

The result of these experiments demonstrates that the influenza bacillus grows well in the emulsion broth described. In this medium *B. influenzæ* was found living and capable of multiplying after 10 days at 37°C. (Table I).

Experiment 1 was repeated in emulsion broth prepared in the manner described from the mucoid growth of an organism classified as *Bacillus ozænæ*.

In this experiment the growth was washed off the surface of plain agar with 1 cc. of normal saline solution and the bacteria were killed by heating at 70°C. for 1 hour. 0.5 cc. of this sterile emulsion was then added to 5 cc. of plain broth. The emulsion broth was inoculated with 0.1 cc. of a blood broth culture of *B. influenzæ* after all blood cells had settled to the bottom of the tube. 24 hours later 0.1 cc. of this first emulsion broth culture was transferred to a second emulsion broth tube and simultaneously streaked on the surface of blood agar to determine growth. This series of cultures was carried on successfully for ten transfers in emulsion broth, and then was voluntarily discontinued. The ten consecutive cultures all showed typical colonies on the blood agar, and films of the last seven transfers showed typical bacilli and a few involution forms of *B. influenzæ*.

TABLE II.

Determination of Smallest Amount of Emulsion Capable of Stimulating Growth of B. influenzæ.

Tube No.	Amount of broth.	Amount of emulsion.	Growth.
	cc.	*cc.*	
1	5	0.5	+++
2	5	0.3	+++
3	5	0.1	+++
4	5	0.01	+
5	5	0	−

Experiment 2.—After it had been shown that an emulsion of heat-killed mucoid bacteria, when added to plain broth, is able to support growth of the influenza bacillus, it seemed desirable to learn how small an amount of the emulsion would suffice for this purpose. Accordingly, dilutions of the emulsion in broth were made and inoculated with comparable amounts of a culture of influenza bacillus. After 24 hours incubation subcultures were made on blood agar to confirm growth in the cultures containing various dilutions of the emulsion. These results are recorded in Table II.

0.01 cc. is evidently the lower limit of the growth-stimulating substance in this particular emulsion of heat-killed mucoid bacilli, since cultures containing this amount showed less growth than those in which larger quantities were used.

Experiment 3.—In order to determine whether the growth of *B. influenzæ* in the emulsion broth was more pronounced in the bottom of the tubes or at the surface of the broth, tubes were inoculated after all mucoid material had settled to the bottom. They were allowed to stand in the incubator for 24 hours and subcultures were made both from the thick residuum in the bottom of the tubes and from the superficial layers of the broth. The growth was compared with the following result.

	Growth.
(*a*) From bottom of tubes	+++
(*b*) From surface	+

The fact that this accessory substance appeared to be more concentrated in the immediate vicinity of the sedimented bacterial emulsion than in the upper portions of the culture fluid seems to indicate that the growth-stimulating factor is contained within the bacterial cell and slowly passes out into the surrounding fluid.

It therefore seemed reasonable to attempt to extract this substance from the bacterial emulsion. The addition of a clear bacterial extract to media, moreover, would have the advantage of making it possible to observe bacterial growth directly without the secondary transfer to blood agar. This was done in Experiment 4.

Experiment 4.—The growth of a mucoid organism was collected from agar plates and emulsified in plain broth, 1 cc. of broth being used to each plate. The emulsion was boiled for 5 minutes, and then centrifuged to separate the clear fluid extract from the bacterial bodies. This sterile extract was then tested for growth-stimulating action by the addition of decreasing amounts to plain broth. The medium prepared in this manner was inoculated with one drop from an emulsion broth culture (Table I, No. 6). The results of this experiment are recorded in Table III.

TABLE III.

Growth-Inducing Action of an Extract of Mucoid Bacteria on B. influenzæ.

Amount of extract.		Growth.
cc.	*per cent*	
0.3	6.0	++
0.1	2.0	++
0.05	1.0	+
0.01	0.2	−
0.001	0.02	−
0	0	−

It is evident from Table III that it is possible by simple boiling of an emulsion of mucoid organisms to obtain an extract which when added to plain broth is capable of inducing growth of *Bacillus influenzæ*. That the first extraction of the bacillary emulsion does not completely exhaust it of this growth factor is shown in the following experiment.

Experiment 5.—An emulsion of mucoid bacilli was made as previously described. A portion of this emulsion was heated at 60°C. for 1 hour and another portion was boiled for 5 minutes and then centrifuged and the clear supernatant extract pipetted off. The bacterial residuum was then washed in normal saline solution three successive times and the following experiment carried out.

	Growth.
5 cc. of plain broth + 0.5 cc. of unboiled emulsion heated to 60°C. for an hour	+
5 cc. of plain broth + 0.5 cc. of extract from boiled emulsion	++
5 cc. of plain broth + 0.5 cc. of residuum from boiled emulsion	++
5 cc. of plain broth (control)	−

In this experiment the extract and the residuum from the boiled emulsion, when added to broth, gave even better growth than the emulsion heated at 60°C. for 1 hour.

That extraction of the growth-inducing substance is obtained simply by allowing the bacterial cells to remain in contact with broth for some time is shown in the following experiment.

Experiment 6.—To two tubes of plain broth there was added 0.5 cc. of a bacillary emulsion which had been heated to 60°C. for 1 hour. The tubes were then left in the ice box for 1 week. After this time, one tube was centrifuged and the clear supernatant fluid used as culture medium, while from the other, the supernatant fluid was pipetted off without being centrifuged. The two tubes were inoculated with 0.1 cc. of an emulsion broth culture of *B. influenzæ*. Good growth occurred in both tubes.

This experiment indicates that the growth-inducing substance passes from the bacterial cells into the surrounding fluid and there exists apart from the cell.

In the foregoing experiments it has been shown that it is possible to obtain good growth of *Bacillus influenzæ* in plain broth to which emulsions and extracts of mucoid bacteria have been added. It seemed reasonable, therefore, to seek the same growth factors in other microorganisms, and *Bacillus proteus* was selected as an organism which normally shows an abundant growth on ordinary media.

Experiment 7.—Agar plates were inoculated with *B. proteus*. After 24 hours growth, to each plate 1 cc. of normal saline solution was added, and the growth washed off and collected in a sterile centrifuge tube. The emulsion was boiled for 5 minutes. The sterile emulsion was then centrifuged and the supernatant fluid pipetted off. This extract was clear, yellowish in color and had a reaction of pH 7.6. 0.5 cc. quantities of this extract were used to enrich plain broth. After the addition of this amount the beef infusion broth remained clear, so that eventual growth could be indicated by the turbidity of the medium. Control cultures, however, were always made from the extract broth on blood agar or oleate hemoglobin agar, and in plain broth.

In the fluid medium thus prepared the following experiment was made. From a 24 hour culture of *B. influenzæ* in blood broth 0.1 cc. was transferred to *Proteus* Extract Broth 1; from this after 24 hours growth, to No. 2, and from this to No. 3. These three cultures all gave good growth and upon transfer to blood agar showed the typical colonies of *B. influenzæ*. Films also showed the typical small Gram-negative bacilli.

The experiment was voluntarily discontinued after the third transfer.

This experiment showed that *Bacillus influenzæ* would grow on a watery extract of *Bacillus proteus* for at least three generations. An experiment was next made with the whole bacterial emulsion of *proteus* in the same manner as described in Experiment 1. Here, however, growth of *Bacillus influenzæ* occurred only in the first two transfers after the blood broth culture. A considerable difference between the emulsions of the mucoid bacilli and the emulsion of *proteus*, therefore, seemed to exist. The explanation of this has not been found as yet, but it is reasonable to seek this in the morphological difference between these two microbes. The large capsule of the mucoid bacilli may be a better growth-inducing factor than the capsule-free *proteus*. The possibility that the capsule may contain some nutritional reserve for the bacillus has already been put forth by Toenniessen (8), who finds that the capsule consists of a polysaccharide, galactan, and that other bacteria grow better on the surface of cultures of Friedländer's bacillus than on plain agar.

Since it had been shown that it was possible to extract a growth-inducing factor from *Bacillus proteus*, tests were made to determine the influence of this extract upon the growth of *Bacillus influenzæ* in various sugar solutions (1 per cent of sugar in peptone water with Andrade indicator (9)).

Experiment 8.—To each 5 cc. of sugar medium was added 0.2 cc. of *proteus* extract. The tubes were then inoculated with 0.1 cc. of culture (No. 2 in the foregoing experiment) and incubated. The following sugars were used: lactose, mannitol, maltose, dextrose, saccharose, raffinose, inulin, and salicin. After 12 hours incubation a heavy clouding of the medium was visible in all tubes and the dextrose culture had turned slightly red. After 24 hours the culture containing dextrose was distinctly red, while those containing the other sugars remained colorless. At this point, control cultures on blood agar from all tubes showed pure growth of *B. influenzæ*. 4 days after inoculation the tubes showed the same reactions. On transfers to blood broth the cultures were all found to be living and pure.

Experiment 9.—It was considered of interest to determine whether or not the clear extract of *B. proteus* could be filtered through a Berkefeld filter without losing its potency. After the extract had been prepared as already described, it was passed through a Berkefeld filter N and the water-clear filtrate, after being proved sterile, was added to plain broth in the following amounts.

Broth.	Filtrate.	Growth.
cc.	*cc.*	
5	1.0	+++
5	0.5	+++
5	0.2	+
5	0.05	−
5	0.01	−
5	0	−

Growth was controlled by turbidity of the medium, by films, and by subcultures on blood agar and plain agar. These controls showed the growing organism to have the characters of *B. influenzæ*.

This experiment shows that the bacillary extract in question can pass through a Berkefeld filter without losing its growth-inducing property.

SUMMARY.

From the data presented in the foregoing experiments it is evident that *Bacillus influenzæ* will grow in a fluid medium consisting of plain broth to which have been added small amounts of emulsions or extracts of mucoid bacilli or of *Bacillus proteus*. The bacterial extracts may be made by simple boiling of the bacillary emulsions in broth or saline solution and centrifuging out the bacterial bodies; they may be filtered without losing their growth-inducing property.

Cultures of *Bacillus influenzæ* in bacterial extract broth, if not too small doses of the extracts were employed, always showed heavier growth than the control cultures in blood broth, and growth occurred at a considerably earlier period than in blood broth. In many instances growth could be seen after 3 to 4 hours, and a bacterial whirl was always visible after 6 hours incubation.

When the nature of the culture used for seeding is not stated, this was 0.1 cc. of the supernatant fluid of a blood broth culture.

All cultures were made in fluid medium; solid medium is much more difficult to use in connection with the extracts.

In explanation of the remarkable growth of *Bacillus influenzæ* in this blood-free medium, the idea is proposed that the growth-stimulating effect of the bacterial extracts is due possibly to substances of the same nature as the so called vitamines.

Further investigations on this principle of bacterial nutrition will appear in subsequent papers, together with a more thorough study of the sources and character of the growth-inducing substances.

CONCLUSIONS.

1. It is shown that *Bacillus influenzæ* will grow profusely in hemoglobin-free media consisting only of plain broth and emulsions or extracts of mucoid bacilli and *Bacillus proteus*.

2. The emulsions and the extracts can be boiled and filtered through Berkefeld filters without losing their growth-inducing property.

3. The growth-stimulating effect of the bacterial extracts is possibly due to substances belonging to the class of the so called vitamines.

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DISSOCIATION OF MICROBIC SPECIES.

I. Coexistence of Individuals of Different Degrees of Virulence in Cultures of the Bacillus of Rabbit Septicemia.

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INTRODUCTION.

It is a general rule to regard the individuals comprising a given strain of a microbic species as identical in nature. So, when a culture is spoken of as virulent, it is supposed to be virulent as a whole, and little or no attention is paid to its individual members. They are tacitly assumed to possess this character in common. As a culture attenuates by passage on artificial media, the formerly virulent germs are again assumed to lose their invasive property simultaneously and uniformly.

This point of view persists in spite of numerous facts that show the assumption of a community of characteristics to be without foundation. Chauveau and Phisalix (1) demonstrated atypical forms of *B. anthracis* in cultures from the lymph glands of animals that had died of this infection. Their *bacille en clou* possessed a morphology distinctly different from that of the typical organism. It was completely avirulent. Elser and Huntoon (2) found that colonies of different appearance existed side by side in freshly isolated cultures of *Micrococcus catarrhalis*, and that one type, increasing with each passage, tended finally completely to supplant the other. Bordet and Sleeswijk (3) found an interesting variation to occur in the behavior of *B. pertussis*. When freshly isolated, this organism grew only upon media enriched with blood, but after several passages it became able to multiply upon ordinary media. The blood microbe had antigenic characters distinctly different from those of the agar microbe. Von Lingelsheim (4) discovered that variants having a peculiar type of colony formation arise in laboratory cultures of *B. typhosus*. His work has been elaborated and

extended to other members of the colon-typhoid group by Gildemeister (5). Baerthlein (6) has demonstrated the simultaneous presence, in single cultures of *B. pyocyaneus*, *Staphylococcus aureus*, and *B. coli*, of individuals giving rise to colonies of different appearance. The "H" and "O" forms of *B. proteus*, first described by Weil and Felix (7), are well known. Eisenberg (8) and Wagner (9) have studied thoroughly the presence of variants in cultures of *B. anthracis*.

The researches of Bordet on phagocytic reactions *in vivo*, following injections of *Vibrio metchnikovii* (10) and of streptococcus (11), have brought out with striking clarity differences in virulence existing among the individuals of one culture. It is remarkable that little stress has been laid on findings of such significance.

With this exception, differences of virulence have received little attention in the above mentioned studies. It has been our good fortune, in the course of studies on the rabbit septicemia bacillus, to observe wide variations in this characteristic, associated with definite difference in growth on fluid media and in colony formation. If all the organisms in these cultures had possessed indistinguishable growth characters, little progress could have been made. As it is, the association of a readily observable criterion with the virulence variation has greatly simplified the study of this problem.

Origin of Materials.

The strains of the rabbit septicemia bacillus used were isolated from spontaneous infections occurring among the normal animal stock of this laboratory. Eight strains were obtained in pure culture from the heart's blood at necropsy. The pathologic picture in six of these cases was that of bronchopneumonia, fibrinopurulent pleuritis, and pericarditis. In the remaining two no gross lesions of any kind were discernible, but the organisms were present in large numbers in the heart's blood.

Method of Isolation.

The heart's blood obtained at necropsy was planted in 10 per cent defibrinated rabbit blood broth. After incubation for 24 hours the cultures were plated out in 5 per cent rabbit serum agar. Isolated colonies were fished to rabbit serum broth. After incubation, growth, and microscopic study, this culture was streaked on 10 per cent rabbit serum agar slants, incubated for 10 hours, and then stored in the ice

chest for 7 days, when it was again transplanted. This routine was rigorously observed for all strains.

Characteristics of the Organism.

The organism under investigation is without doubt one of the pasteurella. It is probably identical with the bacillus of rabbit septicemia first accurately described by Theobald Smith (12). It is a minute non-motile bacillus, occurring singly or in pairs. It is frequently coccoid in shape and in fresh preparation might be mistaken for pneumococcus. It is Gram-negative when stained by Stirling's method. Its bipolar nature is best demonstrated in films from the heart's blood, fixed by heat, and stained for 5 minutes in the cold with Loeffler's methylene blue. The microbes in the films from pleural fluid, tissues, and cultures tend to stain solidly. Pleomorphism is most marked in the condensation water of serum or plain agar slants.

Beef infusion broth and beef infusion agar, pH=7.4, fractionally sterilized, were used as base medium throughout the investigation. All the strains are strongly aerophilic. When first isolated from spontaneous infections they grow very feebly or not at all on ordinary media, but quite abundantly on serum agar or in serum broth. After several transplants on blood or serum agar, good growth occurs on ordinary media, provided that a sufficient quantity of material is carried over. The surface colonies on agar are circular in shape, with even borders. They are whitish and rather opaque at the center, more translucent toward the edges. They are strongly fluorescent, both in daylight and by artificial illumination. In serum and in plain broth the growth is uniformly turbid with little tendency to sedimentation. In tubes of serum broth, inoculated with 0.05 cc. of a 16 hour culture, the lag period is very short and the peak of growth reached by 8 hours incubation. From that time on a rapid decrease in the number of viable organisms occurs.

There is no visible growth on potato. Carbohydrates do not enhance growth. The strains isolated in this laboratory ferment dextrose, levulose, saccharose, and xylose with acid but no gas. The medium used was sugar-free broth to which 1 per cent of carbohydrate was added. The pH before inoculation was 7.5. After incubation at 37°C. for 66 hours, the levulose, dextrose, and saccharose tubes were found to have reached an acidity of pH=5.9 to 6.1. No acid formation was observed in maltose or lactose. In fact, definite alkalinity increase was observed with lactose—pH=8.2. Three strains received through the kindness of Dr. N. S. Ferry behaved as did those just described, with the exception that maltose was fermented.

EXPERIMENTAL.

In the course of virulence studies with strains isolated about 6 weeks previously, the approximate number of organisms injected into the test rabbits was controlled by plating high dilutions of the test culture. Dilutions corresponding to 10^{-9}, 10^{-8}, and 10^{-7} cc. of the serum broth cultures under test were plated in 5 per cent rabbit serum agar. It was thought that plain agar might serve as well for this procedure, since all the strains under test had been growing for some time in artificial media. But upon seeding such dilutions

TABLE I.

Growth of Dilutions of Serum Broth Culture R 15 in Serum and Plain Media.

Dilution of original culture.	No. of colonies on plain agar plate.	No. of colonies on serum agar plate.	Growth in plain broth.	Growth in serum broth.
cc.				
10^{-3}	0	Countless.	−	−
10^{-4}	0	"	−	−
10^{-5}	0	"	+	+
10^{-6}	0	1,500	0	+
10^{-7}	0	194	0	+
10^{-8}	0	21	0	+
10^{-9}	0	2	0	+

+ indicates growth; 0, no growth; −, culture not made.

into plain agar plates, no growth occurred, despite the fact that animals injected with the same dilutions succumbed with typical infections. It was known that good growth could be obtained upon plain agar when a transplant was made of fairly large amounts from tube to tube. It was considered of importance to find out how many organisms it would be necessary to plant in order to obtain growth in medium without serum.

Experiment 1. Growth of Dilutions of a 12 Hour Serum Broth Culture in Serum and Plain Agar and Serum and Plain Broth.—A 12 hour 5 per cent rabbit serum broth culture of Strain R 15 was diluted with plain broth, pH=7.4, to 10^{-9} cc. of the original culture. The dilutions were made as follows: Dilution A, 1 cc. of culture + 4 cc. of broth; Dilution B, 0.5 cc. of Dilution A + 4.5 cc. of broth; Dilution C, 0.5 cc. of Dilution B + 4.5 cc. of broth; and so on, to Dilution I, 0.5 cc. of which would be equivalent to 10^{-9} cc. of the original. Different pipettes

were used for making each dilution. As soon as the dilutions were completed, and starting at dilution 10^{-9} cc., 0.5 cc. was carried over into parallel large tubes of plain and of 10 per cent horse serum agar, which had been melted and cooled to 45°C. The tubes were carefully shaken and the contents poured at once into plates. Such plates were made from all dilutions, from 10^{-9} down to 10^{-3} cc. At the same time, inoculation of the same amount of material was made into parallel series of serum and of plain broth tubes, from dilutions 10^{-9} to 10^{-5} cc. The plates and tubes were incubated at 37°C. for 48 hours. The result is given in Table I.

Table I shows that no growth occurred in plain agar plates, even when these were seeded with 10^{-3} cc. of the culture, an amount containing millions of organisms. Colonies appeared on all the serum agar plates, and in all the serum broth tubes. On the other hand, the plain broth series showed multiplication in 10^{-5} cc., but not in higher dilutions. The most striking fact observed was the character of the growth in the plain broth subculture from dilution 10^{-5} cc. Instead of growing diffusely with a uniform turbidity, as was the case with all the serum broth subcultures, the growth in the plain broth tube was granular in character. These granules were apparent even in early phases of the growth. They settled rapidly to the bottom of the tube, so that in 24 hours the supernatant fluid was perfectly clear.

Repetition of this experiment led to the same result. This phenomenon can be explained in one of two ways. First, it might be conceived that organisms growing diffusely in serum broth subcultures might flocculate when cultivated in plain broth. Or, second, two types of microbe might be supposed to exist in the same culture, one, preponderatingly present, and capable of growing only in serum broth, when seeded in small amount, the other, less numerous, sedimenting in fluid medium, and able to grow in plain broth, even when seeded in relatively small amount. To resolve this question the following experiment was undertaken.

Experiment 2. Persistence of the Growth Character of Granular and Diffuse Growing Microbes.—The plain broth subculture, dilution 10^{-5} cc., of Experiment 1 grew in rapidly sedimenting, granular manner. This culture was seeded in amounts of 0.05 cc. to parallel tubes of plain and of 5 per cent rabbit serum broth. The procedure was repeated through six transfers. The granular growth persisted, in the serum as well as in the plain broth tubes. On the other hand,

it will be recalled that the serum broth subculture, dilution 10^{-9} cc., of Experiment 1, exhibited a diffuse growth. This culture, seeded in similar amount into parallel serum and plain broth tubes, continued to grow diffusely in the plain as well as in the serum broth series.

It would seem, then, that the first of the two possibilities suggested, *i.e.* that of a change of growth character following transfer from serum to plain broth, is answered in the negative. On the other hand, the possibility still remained that the sudden transfer from serum to plain broth might cause a mutation from diffuse to granular growth character, which might persist even after return to serum broth. To rule out this contingency, it was necessary to determine other differences between the granular and diffuse growing microbes, which might lead to the demonstration of their existence side by side in the same culture; that is to say, it was necessary to reveal their coexistence in a given stock culture, without having recourse to dilution into plain broth.

Experiment 3. Difference in Appearance of Colonies of the Granular and Diffuse Growing Microbes.—A stock serum agar slant of Strain R 15 was subcultured into 5 per cent rabbit serum broth. The culture after 6 hours showed diffuse growth. It was diluted to 10^{-3} cc. in plain broth, and subinoculations were made as before into parallel series of plain and serum broth tubes. The serum broth subculture showed diffuse growth in dilutions 10^{-3} to 10^{-2} cc. The plain broth parallel subcultures grew in dilutions up to 10^{-6} cc., but not in dilutions 10^{-7} or 10^{-8} cc. All the tubes of this series were granular in their growth character.

The serum broth subculture, 10^{-3} cc. (diffuse), and plain broth subculture, 10^{-6} cc. (granular), were now transferred into 5 per cent serum broth. The transplant from the first grew diffusely and was designated D. That from the second sedimented rapidly; it was designated G. Cultures D and G were again carried over to serum broth. After 6 hours incubation at 37°C., each tube was diluted in plain broth in the usual manner to 10^{-3} cc. of the original. Tube G was thoroughly shaken to break up the clumps before dilution.

Dilutions 10^{-7} and 10^{-8} cc. of Cultures D and G were plated in 5 per cent serum agar. The surface colonies resulting from Inoculation D were strikingly different from those of Inoculation G. The former were whitish, with rather opaque, glistening centers, fading into translucent outer zones. Their borders were regular. They exhibited marked fluorescence, both by daylight and by artificial light. The surface colonies of Type G were somewhat smaller, translucent, and bluish in color, had irregular serrated edges, and showed little or no fluorescence.

Colonies of the first type, D, fished to 5 per cent serum broth, yielded diffuse growth. Those of Type G gave rise in every instance to granular, rapidly sedimenting cultures.

The fact that the difference in appearance of colonies accompanied the difference in growth character in fluid medium was utilized at once as a means of detecting whether Types G and D exist side by side in strains which have never been carried into plain broth. It will be seen by reference to Table I that the Type G organisms in the original culture are present in smaller numbers than those of Type D. Since, ordinarily, Type G grows in plain broth subcultures from dilutions not higher than 10^{-4} cc., it will be evident that there is no hope of detecting Type G colonies by plating out high dilutions of serum broth cultures. Consequently, the demonstration of coexistence had to be sought by another method.

It was observed that when stock serum agar cultures of the various strains were planted in serum broth the resulting growth was diffuse, but that after 8 to 10 hours incubation there was always a more or less abundant sediment at the bottom of the tube. It was assumed that if the granular organisms were present with the diffuse variety, it would be logical to find them in high concentration in the sediment. This assumption was tested in Experiment 4.

Experiment 4. Demonstration of the Coexistence of Types G and D by Plating the Supernatant Fluids and Sediments of Serum Broth Cultures.—A stock 10 per cent serum agar slant of Strain R 22 was transferred to a tube of 5 per cent rabbit serum broth. After incubation at 37°C. for 24 hours, the tube contained a uniformly turbid supernatant fluid, and in addition a distinct sediment. The supernatant fluid was carefully drawn off by a Pasteur bulb pipette, care being taken not to disturb the sediment. The supernatant fluid was placed in a sterile tube, Tube A.

The sediment was now thoroughly shaken up in 10 cc. of sterile broth—Tube B. Tubes A and B were streaked upon plain and serum agar plates, which were then incubated for 24 hours. The result is summarized in Table II.

The two different type colonies were now fished from the serum agar plates to serum broth. Subcultures from the translucent, nonfluorescent type invariably yielded granular strains. Those from the opaque, fluorescing colonies gave rise to an invariably diffuse growth. Experiment 4 demonstrates clearly that Type G and D microbes

coexist in the stock serum broth tubes. It shows that Type G organisms, usually in the minority, can be easily demonstrated by the separation of the supernatant fluid and the sediment of a serum broth culture, and the streaking of the latter. It must be pointed out in this place that this method of procedure is only necessary when Type D greatly predominates. It has been found that when cultures of the rabbit septicemia bacillus are infrequently transplanted, the more saprophytic Type G tends to gain the upper hand. Under these conditions simple streaking out of a thoroughly shaken culture suffices to place both varieties in evidence.

TABLE II.

Dissociation of Types G and D by Sedimentation.

Tube.	Material.	5 per cent serum agar plate.	Plain agar plate.
A	Supernatant fluid of 24 hr. serum broth culture, Strain R 22.	Good growth; both types present. Type D (fluorescent) preponderates.	Scanty growth; both types present, but Type D preponderates.
B	Sediment of 24 hr. serum broth culture, Strain R 22.	Good growth; both types present. Type G (non-fluorescent) largely preponderates.	Very scanty growth; almost entirely non-fluorescent Type G.

Up to the present three differential characteristics of Types D and G have been discussed. (1) Type G is able to grow much more easily than Type D in plain broth; that is to say, much larger amounts of Type D than of Type G are necessary to cause multiplication in this medium. (2) Type G grows in minute granules in liquid media, these particles sedimenting rapidly and leaving the supernatant fluid clear. Type D, on the other hand, grows diffusely, both in serum and in plain broth. (3) The colonies produced by Microbe D are distinctly different from those of Type G.

Careful study has revealed no difference in morphology or tinctorial reaction between Types G and D. Their fermentation reactions have proved to be identical. The question arises whether other differences exist.

Since Microbe G grows with distinctly greater ease in plain broth, it is easy to imagine that this fact might represent an adaptation of the organism, ordinarily rather delicately adjusted to the parasitic state, to a saprophytic existence; that is to say, Type G, which is more saprophytic, might exhibit a corresponding loss in parasitic activity, and hence might be of decidedly less virulence for rabbits than the corresponding Type D.

This hypothesis is found to be vindicated in a striking manner in Experiment 5.

TABLE III.

Comparative Virulence of Types G and D, Strain R 15.

Type.	No. of passages after dissociation.	Weight of rabbit.	Age of culture.	Amount injected intra-pleurally.	Result.
		gm.	*hrs.*	*cc.*	
D	Serum broth third passage.	550	6	10^{-7}	Negative.
		550		10^{-6}	Died in 42 hrs.*
		550		10^{-5}	Died in 42 hrs.*
G	Plain broth first, serum broth second passage.	550	6	10^{-7}	Negative.
		550		10^{-5}	"
		550		10^{-3}	"

* Autopsy typical; fibrinopurulent pleuritis, pericarditis, and bronchopneumonia. Pure culture of Type D from heart's blood.

Plate of dilution 10^{-8} cc., Type G, 32 colonies.

Plate of dilution 10^{-8} cc., Type D, 21 colonies.

Experiment 5. Comparative Virulence of Types D and G.—Microbe D was obtained by transplanting a diffuse growing serum broth subculture from dilution 10^{-8} cc. of a 6 hour serum broth culture of Stock Strain R 15. Third passage in serum broth. Microbe G was obtained by transplanting a granular plain broth subculture from dilution 10^{-6} cc. of the same serum broth culture of Strain R 15. Passage in serum broth exactly parallel to that of Microbe D.

Serum broth cultures of each variety were incubated for 16 hours and seeded in 0.05 cc. amounts into tubes of 5 per cent rabbit serum broth. Incubation for 6 hours at 37°C. Dilution in plain broth to 10^{-8} cc., care being taken to disintegrate thoroughly the granules of Culture G. Culture D was then injected intrapleurally, in doses of 10^{-7}, 10^{-6}, and 10^{-5} cc., into rabbits of 550 gm. weight.

Culture G was injected by the same route into rabbits of the same weight in doses of 10^{-7}, 10^{-5}, and 10^{-3} cc. Dilutions of 10^{-8} cc. of each culture were plated in 5 per cent serum agar. The result, which was most striking, is recorded in Table III.

Repetition of this experiment with the same and with other strains yielded constantly a like result. In a word, Microbe G, recently dissociated from a stock strain, shows itself to be distinctly less virulent than its companion Type D of the same strain. All experiments of this type were made with organisms dissociated from the same strain, at the same time, and transplanted under rigidly parallel conditions. The results in Table III are by no means the most striking obtained, since young rabbits frequently resist 0.1 cc. of a 6 hour culture of Type G, while parallel Type D cultures are often fatal in dose of 10^{-7} cc. and never in less than 10^{-4} cc.

TABLE IV.

Virulence of Types D and G, Strain R 22, Dissociated by the Sedimentation Method.

Type.	Strain.	No. of passages in serum broth.	Age of culture.	Amount injected intra-pleurally.	Result.
				cc.	
G	R 22	7	6 hrs., 1,100,000,000 colonies per cc.	10^{-4}	Negative.
				10^{-3}	"
				10^{-2}	"
				10^{-1}	"
D	R 22	7	6 hrs., 1,140,000,000 colonies per cc.	10^{-6}	"
				10^{-5}	Died in 16 days.*
				10^{-4}	Died in 4 days.*
				10^{-3}	Died in 39 hrs.*

* Autopsies in all cases typical. Pure Type D recovered from heart's blood of each animal.

It might be objected in the experiment just described that the Type G organisms had lost their virulence by reason of their transplantation, for one passage, in plain broth. The method of dissociation by sedimentation described in Experiment 4 makes it easy to

examine into the validity of such an objection. This technique was therefore applied to Strain R 22, and the virulence of the resulting Type G and D varieties tested. In this case Strain G had received no passage through plain broth.

Experiment 6. Virulence of Types D and G, Strain R 22, Dissociated by the Sedimentation Method.—In order to insure the purity of each type, three successive platings in serum agar, with alternate transfers into serum broth, were made, after the primary separation by sedimentation. After the third plating, Microbes D and G were carried for three passages, daily transfer, in serum broth. Each was then subinoculated from a 12 hour serum broth culture into a tube of 5 per cent rabbit serum broth. Incubation at 37°C. for 6 hours; dilution, as usual, to 10^{-7} cc. of the original culture. Type G was then injected into 1,500 gm. rabbits, intrapleurally, in doses of 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} cc. Type D was injected by the same route into rabbits of the same size, in doses of 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} cc. The result is given in Table IV.

It must be stated here that the infection in rabbits of large size is identical in nature with that of young animals, the sole difference being in the time of death.

Experiments 5 and 6 show clearly that Microbe G is distinctly less virulent than Microbe D. It was natural to ask whether Microbe G could produce typical infections when injected in overwhelming quantities. It was found that adult rabbits tolerated doses of 0.5 cc. of whole serum broth culture. Young rabbits were for this reason selected for the experiment.

Experiment 7. Effect on Young Rabbits of Massive Doses of Type G.—A series of 600 gm. rabbits was injected intrapleurally with 3, 2, 1, 0.5, and 0.1 cc. of a 6 hour culture of Strain R 15, Type G. The rabbit injected with 3 cc. succumbed in 5 days. Those that received 2 and 0.5 cc. died in 32 and 24 hours, while doses of 1 and 0.1 cc. produced no lethal effect. It is important that the organisms recovered at necropsy showed a granular growth in serum broth, and when streaked upon serum agar plates gave rise to typical Microbe G translucent colonies.

The pathologic effects produced by Microbe G as compared with those produced by the more virulent Microbe D will be dwelt upon in a later communication in which the possibility of raising the virulence of Type G will be discussed. It is enough to say that this microbe retains its character of growth after one passage through the animal body.

It is frequently remarked that the virulence of many organisms is lost more quickly by cultivation in ordinary media than in media enriched with serum or blood. Observations have shown that with Type D (virulent) of various strains of the organisms under consideration, the characteristic diffuse growth is retained despite many passages in plain broth. Since it has been noted that the characters of diffuse growth and virulence in this instance accompany one another, it was considered important to discover whether plain broth passage would depress the virulence of Microbe D, while the diffuse growth character remained. Experiment 8 supplies an unequivocal answer to this question.

Experiment 8. Comparative Virulence of Type D, Strain R 15, after Twenty-Five Parallel Passages in Plain and Serum Broth.—Microbes D and G were dissociated from Stock Strain R 15 by the first of the methods described above; *i.e.*, by dilution and parallel subculture in plain and serum broth. They were plated three successive times in serum agar, isolated colonies being fished to serum broth each time. This insured as nearly as possible that the strains arose from a single organism. Microbe D was then carried in parallel passage through twenty-five daily transplantations in plain and in serum broth. From time to time the cultures were streaked on serum agar plates. With both the plain and the serum broth tubes organisms of Type D only were invariably found. What is more, the plain broth strain retained perfectly its characteristic of diffuse growth. The plain broth passage strain is designated as D–Pl, that of the serum broth passage as D–S.

Microbe G, dissociated at the same time, was subjected to a similar number of passages in serum broth. The virulence of all three of the strains was now tested. 6 hour serum broth cultures of Microbes D–S, D–Pl, and G–S were diluted in plain broth to 10^{-7} cc. of the original cultures. The injection of the test animals was carried out immediately after the dilution of each culture. At the end of the injections plates were in each instance made in 5 per cent serum agar. The result is summarized in Table V.

Table V shows very clearly that plain broth passage has little or no effect upon the virulence of Microbe D. On the other hand, Microbe G characteristically fails to kill in 0.1 cc. Up to the present time we have been unable to discover Type D varieties possessing low virulence in any of our strains. What is more, it is a remarkable fact that Strains R 19 and R 21, which so far have shown no evidence of organisms of Type G, are by far the most invasive of any of the

strains studied. This experiment, together with other observations described above suggest that the mechanism of attenuation may be a selection; that is, an overgrowth of the virulent Type D by the less virulent Type G. This hypothesis is amenable to experimental proof and will be discussed fully in a later communication.

TABLE V.

*Comparative Virulence of Type D, Strain R 15, after Twenty-Five Parallel Passages in Serum and Plain Broth.**

Type.	Strain.	No. of passages.	Weight of rabbit.	Age of culture.	Amount injected intra-pleurally.	Result.
			gm.	*hrs.*	*cc.*	
D	R 15	Serum broth, twenty-five passages.	700	6	10^{-6}	Died in 24 hrs.†
			725		10^{-5}	" " 36 " †
			650		10^{-4}	" " 42 " †
			675		10^{-3}	" " 38 " †
D	R 15	Plain broth, twenty-five passages.	700	6	10^{-6}	" " 36 " †
			750		10^{-5}	" " 24 " †
			750		10^{-4}	Negative.‡
			700		10^{-3}	Died in 31 hrs.†
G	R 15	Serum broth, twenty-five passages.	700	6	10^{-4}	Negative.
			700		10^{-3}	"
			650		10^{-2}	"
			675		10^{-1}	"

* Microbe G–S 25, control.

† Autopsy typical; fibrinopurulent pleuritis, pericarditis, and bronchopneumonia. Pure cultures of Type D from heart's blood.

‡ Probably technical error in inoculation.

Number of colonies in 10^{-7} cc.: Microbe D–S, 30; Microbe D–Pl, 53; Microbe G–S, 10.

Immunologic Relations of Types G and D.

Microbes G and D in all instances so far studied differ in growth character in fluid media, in colony formation, and in virulence. It is logical to inquire into their immunologic relations. The first experiment consisted in determining the degree of resistance offered by animals which had survived injection with Microbe G to injections of multiple lethal doses of the virulent Type D.

Experiment 9. Resistance of Animals Which Had Survived Injection with Type G to Multiple Lethal Doses of Type D.—Rabbits 1 and 2, weighing 1,500 gm. each, withstood with no visible effect 0.1 and 0.5 cc. of Type G, Strain R 15, injected intrapleurally. 14 days later they were injected by the same route with 10^{-4} cc. of a 6 hour serum broth culture of the virulent Type D, Strain R 15. Immediately afterward, controls of the same weight received doses of 10^{-6}, 10^{-5}, and 10^{-4} cc. of the same culture. The result is summarized in Table VI.

As seen from Table VI, Rabbits 1 and 2 were resistant to at least 100 lethal doses of the virulent Strain D. It is of interest that such solid immunity should be conferred by a single injection of the less virulent Strain G. Other experiments gave the same result. It

TABLE VI.

Resistance of Animals Which Had Survived Injection with Type G, Strain R 15, to Multiple Lethal Doses of Type D, Strain R 15.

Rabbit No.	Amount of Type G injected intrapleurally 14 days previously.	Weight of rabbit.	Amount injected for test of Type D.	Result.
	cc.	*gm.*	*cc.*	
1	0.1	1,500	10^{-4}	Negative.
2	0.5	1,500	10^{-4}	"
3		1,450	10^{-6}	Died in 9 days.*
4		1,500	10^{-5}	" " 4 " *
5		1,475	10^{-4}	" " 4 " *

* Autopsy typical. Pure Type D recovered from heart's blood.
Number of colonies on serum agar plate of dilution 10^{-8} cc., 16.

must be remarked that no experiments have been instituted that would indicate the comparative immunizing efficacy of the virulent Type D and the less virulent Type G.

Agglutination Reactions of Types G and D.

The vaccinating power of Type G against the virulent Type D has been clearly demonstrated in Experiment 9. The results of agglutination studies bear out the community of antigenic quality suggested by the vaccination experiments. Stable suspensions of Microbe G (granular) may be prepared by washing the organisms from 5 per cent serum agar slants four times in distilled water, and

finally suspending them in this medium. 0.1 per cent formaldehyde was added to all suspensions.

The flocculation of both Types G and D by immune sera is very slow and is much more clear-cut at 55° than at 37°C. The necessity of incubation at 55° is especially marked with Type D. This fact is, indeed, of differential value, since Type G suspensions agglutinate very well when placed in contact with immune serum at 37°C. for 16 hours. Parallel tests made on Microbe D at the same temperature do not give satisfactory results.

Serum prepared by three intravenous injections of Microbe D into rabbits agglutinates Types D and G in titer of 1:2,000 after incubation at 55° for 16 hours. A serum resulting from similar injection of Microbe G is considerably less active, agglutinating Type G in 1:1,000 and Type D in 1:200. In some instances Microbe D agglutinated in dilution of 1:50 but not in higher dilutions. To sum up, the antigenic power, as far as the production of agglutinin is concerned, appears to be decidedly stronger in the case of Type D than of Type G.

Absorption tests clearly indicate the community of antigenic character of the two types. Serum > Type D, Strain R 15, agglutinated Microbes D and G in titer of 1:1,500. After 2 hours contact at 55° with a suspension of Microbe D, the titer for Type G had fallen to 1:200, for Type D to 1:80. The control serum, after 2 hours at 55° without the suspension, showed the original titer, 1:1,500. The same serum, placed for a similar length of time in contact with Microbe G, dropped in titer from 1:1,500 to 1:40 for Types D and G. Various observations make it apparent that Microbe G, in addition to being more easily flocculable, also has greater binding power for the agglutinating principle than has Microbe D. From the foregoing results it is apparent that there is no qualitative difference in the antigenic nature of Types G and D. In a later communication these agglutination reactions will be dealt with more fully, and the acid flocculability of the two types will be discussed. The acid agglutination points of Types D and G are distinctly different. They are of the nature of physical constants, and hence present a valuable differential criterion.

SUMMARY AND CONCLUSIONS.

Two types of organism have been shown to exist in cultures of the bacillus of rabbit septicemia, recently isolated from spontaneous infections.

One, Microbe D, grows diffusely in serum and plain broth, forms rather opaque, fluorescing colonies on serum agar, and is highly virulent for rabbits. These characters are retained throughout many passages in serum or plain broth.

The other type, Microbe G, flocculates rapidly in fluid media, forms translucent, bluish colonies with little fluorescence, and exhibits extremely low virulence for rabbits. Like Microbe D, its distinguishing characters persist throughout many passages in artificial media.

Two methods for the dissociation of these varieties from the parent culture have been described.

The two types are morphologically indistinguishable and possess identical fermentation reactions.

Rabbits surviving inoculation with Type G are resistant to multiple lethal doses of Type D. The agglutination reactions bear out this suggestion of the antigenic identity of the varieties. Community of antigenic character is rendered certain by the results of absorption reactions.

Microbe D, in contact with immune serum, flocculates well at 55°, but poorly or not at all at 37°C. Microbe G, on the other hand, agglutinates easily at both temperatures.

Microbe D, after being carried through twenty-five passages in serum and in plain broth, retains perfectly its characteristics of diffuse growth and of virulence, in the plain as well as in the serum broth.

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STUDIES ON DECREASING THE REACTION OF NORMAL SKIN TO DESTRUCTIVE DOSES OF X-RAYS BY PHARMACOLOGICAL MEANS AND ON THE MECHANISM INVOLVED.*

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Plates 100 to 102.

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In an earlier investigation evidence was presented to show that an organism sensitized by a foreign protein could locally autoinoculate itself with the same protein when certain conditions were fulfilled.[1] As this mechanism would serve to explain a number of abnormal reactions of hitherto cryptogenetic origin, it was desirable to advance still more proof. For this reason work was undertaken in which x-rays were the local irritating agent which caused the autoinoculation. During the preliminary stages of this research, however, an impression gradually developed that a certain group of rabbits seemed to show an increased resistance to doses of ordinarily destructive x-rays. Since this would be of considerable theoretical as well as practical value, if true, we abandoned our original object temporarily in order to study this point. The result demonstrated the correctness of the impression that the skin after the systemic incorporation of serum could be rendered resistant to doses of x-rays which are lethal to the tissues of non-prepared animals.

*A preliminary statement was recently published (Auer, J., and Witherbee, W. D., *J. Am. Med. Assn.*, 1921, lxxvi, 301).

[1] Auer, J., *J. Exp. Med.*, 1920, xxxii, 427.

Method.

An interruptorless, 10 kilowatt, 220 direct current machine with a medium focus Coolidge tube was used. After preliminary trials 30 skin units (Witherbee-Remer formula)[2] were chosen as the standard test dose of x-rays. This was produced by a 3 inch spark-gap, 10 milliampere current, 6 inch distance from target, and 20 minute exposure. All these factors were constantly controlled throughout the period of treatment of all the animals. No filter was employed, except that a disc of ordinary filing card was placed between the tube and the skin surface in order to reduce the heat effect.

Rabbits only were used. The area x-rayed was always 4 sq. cm. of the upper half of the right ear, the central artery of the ear passing through the middle of this space. The rest of the ear and body was protected by a sheathing of lead. Shifting of the x-rayed area, due to movements of the animal, was minimized by a simple device. The right ear was turned forward, smoothed out upon a small board and a strip of plaster fixed the tip of the ear to the board and the board to the box. A mask of sheet lead provided with an opening of 2 by 2 cm. was carefully placed in position on the right ear and held there by a strip of plaster. After covering the rest of the head and the entire box with lead sheeting, the animal was ready for treatment. Great care was exercised to prevent a circular constriction of the neck. This procedure was quite successful, though a moderate lateral shifting of the ear occurred in some instances.

It should be emphasized that the site chosen for x-raying offers a number of advantages: the ear is always easily available for inspection with no discomfort to the animal; two skin surfaces are affected by the x-rays, the dorsal on entry and the internal surface on exit of the x-rays; no serious systemic effects are to be feared even after massive doses because the x-raying is entirely localized to a comparatively small area; the ear of the rabbit is richly vascularized, and possesses a number of direct arteriovenous anastomoses[3] which guarantee an especially efficient collateral circulation.

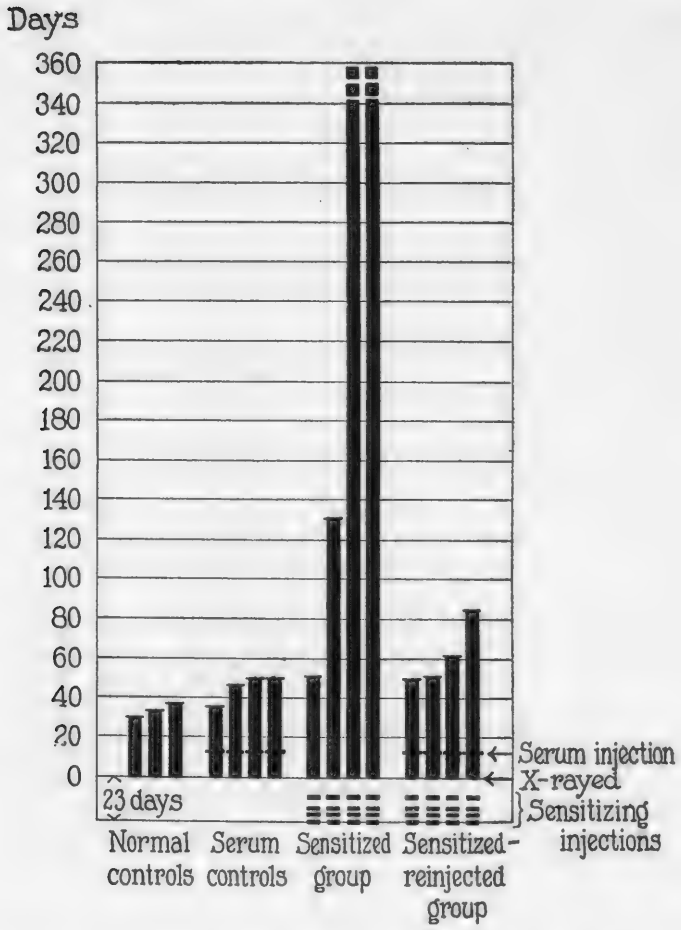
All animals except the normal controls were x-rayed on the same day, but the members of no group were x-rayed in succession. The procedure was to take one animal from each group in rotation until all animals had been exposed to the x-rays.

After the rabbits had been x-rayed they were examined at 2 to 4 day intervals or daily when necessary, for a period of over 300 days, at which time the evidence was deemed sufficient to terminate this aspect of the work.

The end-point of the reaction was the appearance of a spot of dry gangrene in the x-rayed area, with subsequent fenestration. The number of days which elapsed between the time of x-raying and the appearance of dry gangrene, or the

[2] Witherbee, W. D., and Remer, J., *Arch. Dermatol. and Syphilol.*, 1920, i, 558; *Am. J. Roentgenol.*, 1920, vii, 485.

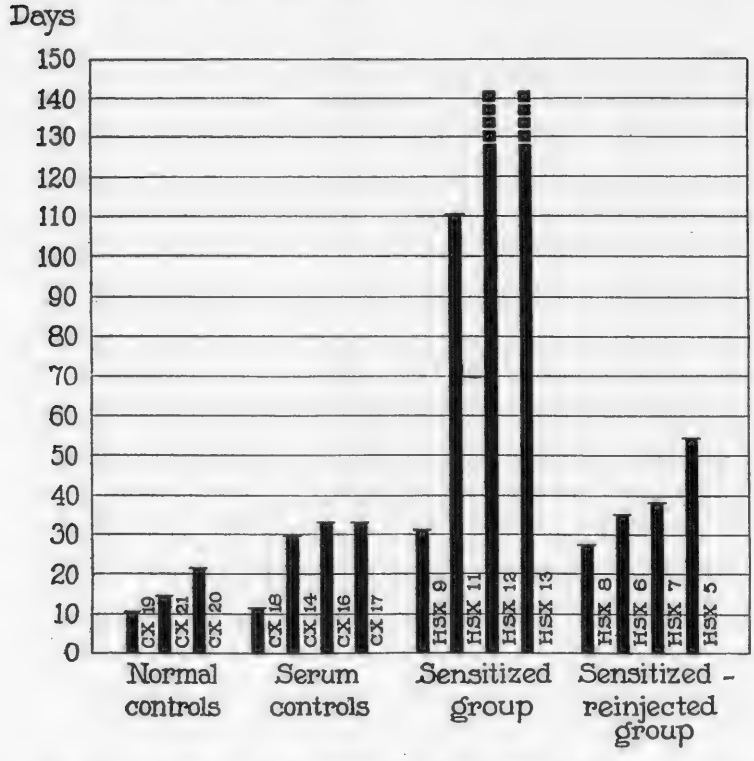
[3] Berlinerblau, F., *Arch. Anat. u. Physiol.*, 1875, 177.



TEXT-FIG. 1. Duration of the reaction from the day of x-ray treatment to the appearance of perforating gangrene.

duration of the inflammatory process up to gangrene, was then plotted. Text-figs. 1 and 2 bring out well the striking difference between the various groups.

The type of rabbit employed, their feeding, care, method of injection, etc., have been described in an earlier paper.[1]



TEXT-FIG. 2. Duration of the reaction from the onset of the first exudate to the appearance of perforating gangrene.

The experimental animal material was composed of four groups of rabbits: (1) normal controls, (2) serum controls, (3) sensitized group, and (4) sensitized-reinjected group.

The normal controls were normal, untreated rabbits in which various doses of x-rays were tested as described. Both ears were utilized at different times. There were six rabbits in this group; in three the standard dose was used.

The serum controls, five in number, were normal rabbits which received a single injection of 10 cc. of horse serum[4] intraperitoneally, 13 days after the ear had been x-rayed.

The sensitized group of rabbits, five in number, was sensitized by two subcutaneous and two intramuscular injections of 1 cc. of horse serum each, at 3 to 4 day intervals. 10 days after the last sensitizing dose the right ears were x-rayed locally with the standard dose.

The sensitized-reinjected group, five rabbits, was prepared exactly as has been described for the sensitized group, but 13 days after x-ray treatment this group was reinjected intraperitoneally with 10 cc. of horse serum. This group was thus subjected to an anaphylactic reaction 23 days after the last sensitizing dose of serum. Text-fig. 1 shows the relation of the groups and the various procedures.

During the early stages of the work one rabbit in each of the last three groups died without obvious lesions. These groups therefore now consisted of four rabbits each.

RESULTS.

Before presenting the results in detail we shall first give the main outstanding facts of this work.

The normal control rabbits developed dry gangrene and fenestration in the x-rayed area in 30, 33, and 37 days respectively.

The serum control rabbits showed dry gangrene and fenestration in the x-rayed ears after 36, 47, 50, and 50 days respectively (Figs. 1 and 2).

The sensitized-reinjected group exhibited the same lesions after 50, 52, 62, and 85 days respectively (Figs. 5 and 6).

The sensitized group, however, responded quite differently on the whole. Only one rabbit showed gangrene and fenestration of the ear in 46 days. A second rabbit developed the same lesion, but only after 131 days. The two remaining rabbits have developed no gangrene or fenestration even after the lapse of more than 340 days (Text-figs. 1 and 2 and Figs. 3 and 4).

These data demonstrate clearly that rabbits previously sensitized by the parenteral injection of horse serum acquire a remarkably increased resistance in the majority of instances to doses of x-rays which are lethal to the tissues of normal control rabbits or serum control rabbits.

[4] The horse serum was kindly furnished by Dr. W. H. Park and Dr. E. J. Banzhaf of the Department of Health of the City of New York.

The results also show convincingly that the protection which serum sensitization previous to x-ray treatment confers is largely abolished when the sensitized and x-rayed animals are subjected to a general anaphylactic reaction (Text-figs. 1 and 2, sensitized-reinjected group).

The main objective details of the investigation are as follows: Within 24 hours after x-ray treatment two to three rabbits out of each group of five showed a slight pinkness of the x-rayed area which disappeared within 2 to 3 days. This pinkness is probably due to a heat effect from the Coolidge tube. Within 4 days the hair of the x-rayed area began to loosen, though there was considerable variation. Thus for example on the 11th day after x-ray treatment some ears showed bald patches, while in others the hair was still firmly fixed. This variation bore no relation apparently to the experimental group. Pigmentation of the x-rayed area was noticeable in 2 to 4 days after x-ray treatment and varied with the different animals. In some it became very marked, while in others the pigmentation was always slight. The degree of pigmentation bore no definite relation to the group to which the animal belonged. A slight thickening of the x-rayed area, without any obvious vascular congestion, was first noticed 9 days after x-ray treatment; it occurred in one to three rabbits of each group. After 11 days these rabbits showed, in addition to the thickening, a slight but definite congestion of the x-rayed area. 13 days after x-ray treatment all rabbits, except two members of the sensitized-reinjected group, showed a definite though slight congestion with slight thickening of the x-rayed area. It should be noted that no rabbit had yet been reinjected with serum at the time of this examination.

The congestion of the x-rayed area increased slowly, but not at an equal rate in all the groups. Thus, 16 days after x-ray treatment the serum control rabbits still exhibited only a slight congestion of the x-rayed area, while the sensitized group showed a moderate to marked congestion, and the sensitized-reinjected animals a fair to moderate congestion. Associated with the increased congestion there was also a slight increase in the thickness of the x-rayed patch.

After 18 to 25 days generally the x-rayed area developed an exudate on both surfaces which dried into crusts. The healing of this first inflammation was usually complete within 28 to 36 days and the x-rayed areas now appeared like healed superficial wounds. The x-rayed area was absolutely bald and practically free of crusts; the skin was thin, whitish, glistening, and easily crinkled into thin folds; the blood supply was good, though many rabbits showed pearly white spots in the x-rayed areas; there was no gangrene.

This termination of what we shall call the first inflammation did not take place in all rabbits, but occurred in a majority of the serum controls, the sensitized group, and the sensitized-reinjected group (Table I). In the normal controls (three rabbits) this first inflammation with crusts did not clear up but passed at once to a complete perforating gangrene. The same fact was also observed once in the serum control group (No. CX 18) and once in the sensitized-reinjected group (No. HSX 8) (Table I).

TABLE I.

Group.	Series No.	First inflammation.		Interval between first and second inflammation.	Second inflammation.		Delayed second inflammation.	Length of observation.
		Day of onset of exudate.	Day of healing.		Day of onset of exudate.	Day of appearance of gangrene.		
				days				*days*
Normal controls.	CX 19	22nd	No healing before gangrene.			33rd	—	69
	CX 20	16th	" "	" "		37th	—	69
	CX 21	16th	" "	" "		30th	—	69
Serum controls.	CX 14	18th	28th	8	36th	46th–49th	0	300+
	CX 16	18th	36th	6	42nd	49th–52nd	0	300+
	CX 17	18th	36th	13	49th	49th–52nd	0	300+
	CX 18	25th	No healing before gangrene.			36th	171st–195th	300+
Sensitized group.	HSX 9	15th	28th	14	42nd	46th	0	300+
	HSX 11	20th	28th	68	96th	128th–135th	0	300+
	HSX 12	20th	36th	At least 340.	0	0	0	300+
	HSX 13	23rd	28th	At least 340.	0	0	0	300+
Sensitized and reinjected group.	HSX 5	31st	42nd	10	52nd	82nd–89th	0	300+
	HSX 6	16th	36th	13	49th	49th–52nd	0	300+
	HSX 7	25th	49th	3	52nd	61st–64th	0	300+
	HSX 8	25th	No healing before gangrene.			52nd	0	300+

The figures represent the number of days after x-ray treatment unless otherwise stated. In all rabbits 4 sq. cm. of the right ear were x-rayed with 30 skin units.

The recovery from the first inflammation and the disappearance of the crusts were, however, not permanent in all the rabbits. After a period during which the x-rayed areas looked like healed or practically healed surface wounds, another exudate appeared unexpectedly on these x-rayed surfaces. This typical, second inflammation always led to a perforating gangrene (Table I). The interval elapsing between the end of the first inflammation and the onset of the second inflammation varied from 3 to 13 days in the serum controls and the sensitized-reinjected group. In the sensitized group, on the other hand, the interval before the second inflammation appeared was 14 days in one rabbit, 68 days in a second, and in the two remaining rabbits no second inflammation leading to gangrene has developed after more than 340 days (Table I and Text-fig. 1).

The character of the second inflammation occurring in the x-rayed area was interesting. A fairly marked redness and swelling of the x-rayed tissue preceding gangrene were observed only once (No. HSX 11), and in all the other rabbits in which fenestration of the ear developed, the inflammatory signs were a moderate pinkness of the x-rayed tissue with no definite swelling or heat; the most noticeable feature was the appearance of a sticky exudate, often on both ear surfaces. This exudate apparently was poured out at various rates, for the subsequent crusts exhibited a definite lamination[5] in many instances.

The difference between the inflammatory reaction of the x-rayed tissue and the normal surrounding tissue was well illustrated in four animals, one in each group—Nos. CX 21, CX 14, HSX 11, and HSX 8 (Table I). In these rabbits an inflammation of the right ear set in, perhaps due to scratching. This inflammation was most marked around the periphery of the x-rayed area, especially at the upper and lower borders. In the untreated part the tissues were red, swollen, hot, and in two rabbits (Nos. HSX 11 and HSX 8) a tongue of edema ran from the lower border of the x-rayed area towards the base of the ear; the blood vessels of the untreated part of the ear were markedly dilated. The inflamed tissue, however, stopped sharply at the x-rayed area, and this latter tissue, while more or less pink in all, stood out in striking contrast to the inflamed surroundings, which thus framed the comparatively pallid x-rayed area. The central artery, turgid with blood above and below the x-rayed area, was a mere red thread within this space. There was no appreciable thickening of the x-rayed area on palpation except in No. HSX 11, in which a well marked edema of the lower portion set in. The second inflammation of the x-rayed area was therefore in general of a definite subacute character, while the first inflammation resembled a mild, acute inflammation.

Another striking difference between the x-rayed and normal tissues was observed in the development of exudate and crusts in the four rabbits mentioned above. The marked inflammation of the normal ear tissue did not lead to exudate and crust formation, while thick crusts often developed in the x-rayed areas.

[5] This lamination was observed in the secondary exudate; no notes were made on the structure of the crusts in the primary exudate.

The chief sign which heralded the onset of a perforating gangrene was the appearance of a small, brown-black, slightly sunken spot on the internal skin surface of the x-rayed area; occasionally an exceptionally thick crust was the first sign. In the last three groups the sunken, discolored spot was noted seven times in the ten rabbits in which fenestration took place. The appearance of this discoloration, however, did not invariably foretell the onset of a perforating gangrene. Thus No. HSX 13 exhibited a brown discoloration of the internal surface with slight, thin crust formation 64 days after x-ray treatment. This lesion did not progress, but was practically healed on the 86th day. It will be remembered that this rabbit belongs to the sensitized group and showed no gangrene of the x-rayed area within more than 340 days after x-ray treatment (Table I and Text-fig. 1).

The initial point where a perforating gangrene developed was small when first observed, at times not more than 1 to 2 mm. in diameter. This dry spot then increased in size, first rapidly, then slowly until an equilibrium was established between the destructive and reparative factors. Several times two spots of gangrene developed, one on each side of and close to the central artery of the ear. These two spots always fused sooner or later, but the gangrenous process was always more rapid away from the artery than towards it, though finally the intervening section of the artery also dried up.

The amount of tissue lost by gangrene was never equal to the entire area x-rayed; in only two instances did the loss exceed 50 per cent. The gangrenous process began near the center of the area x-rayed and then progressed towards the borders. This spread was usually greater in the lateral direction than towards the root or apex of the ear. In the serum control group the loss of tissue varied between 80 and 130 sq. mm.;[6] in the sensitized group (two rabbits) between 117 and 224 sq. mm.; and in the sensitized-reinjected group the loss fluctuated between 70 and 210 sq. mm.

The measurements given are only rough approximations of the losses, and no effort was made to determine the true areas of the more or less irregular fenestrations. We believe, however, that the figures convey a just impression.

In addition to the second, subacute type of inflammation leading to gangrene, which has been described, a delayed, second form of subacute inflammation also leading to gangrene may be distinguished. This form was observed only once; it occurred in Rabbit CX 18 of the serum control group (Table I). In this rabbit the first inflammation beginning 25 days after x-ray treatment led at once to a perforating gangrene 36 days after treatment. A similar acceleration of the process took place in No. HSX 8 of the sensitized-reinjected group and in all the

[6] No measurements were made in the last rabbit, No. CX 18, because the gangrene involved the border of the ear, due to a shift during x-ray treatment. Here also the loss was less than 50 per cent of the x-rayed area. The loss of tissue sustained by the normal controls was not measured, due to an oversight.

normal controls (Table I). In No. CX 18, however, a subacute inflammation leading again to gangrene developed 135 days after the first. During this interval of time the remainder of the x-rayed area did not exhibit any obvious differences from the x-rayed areas of other rabbits.

Final Changes in the X-Rayed Area.

When the x-rayed areas of all the rabbits are examined some months after the last inflammation, all of them, including Rabbits HSX 12 and HSX 13 which never developed a perforating gangrene, show a number of changes in common. In all, the x-rayed area is hairless, the skin covering this area is smooth with perhaps a slight branny desquamation on the dorsal surface, and this skin wrinkles readily into thin folds. Occasionally, especially on the internal surface, numerous small, oval, yellowish brown thickenings of the outer skin are observable. These are less than 1 mm. in diameter and still less in thickness. They are seated in cup-shaped depressions of the skin, and probably represent keratoses.

The borders of the fenestrations generally show little or no thickening, but at or near the fenestration one or more red or reddish brown, slightly elevated papules are observable. These papules are formed by a number of dilated, small blood vessels. Occasionally, a slight hemorrhage proceeds from the angiectasias and the blood may burrow under the outer layers of the skin epithelium. These small masses of dilated blood vessels were also observed in Rabbit HSX 13 in which no perforating gangrene occurred; they were not seen in Rabbit HSX 12 of the same group.

In addition to these angiectasias the x-rayed areas show a number of tortuous blood vessels; often they are especially evident upon the internal surface. In the two rabbits of the sensitized group, Nos. HSX 12 and HSX 13, in which gangrene of the x-rayed area did not develop, these tortuous blood vessels are especially noticeable about the neighborhood of the central artery, where they form a delicate tracery of blood channels which are apparently superficial. The central artery itself in the x-rayed area of these two rabbits is narrow, slightly irregular in outline, and looks blurred in that portion of its course where the angiectasias are most marked.

The blood vessels of the healed, x-rayed areas do not react normally. In the normal rabbit the ear vessels respond by an initial blanching when the animal is sharply tapped, or a moderate struggle is induced; this blanching is followed by a marked vasodilatation if the original stimulus was sufficiently strong and if the room is not too cold. In the x-rayed areas of the experimental rabbits, however, this test causes at first some increase in the pallor, which later is replaced by only a slight dilatation of both arteries and veins. This striking difference in the vasomotor response of the x-rayed and untreated ears is well illustrated by Figs. 1 to 6, which were obtained by photographing the ears of two members of each group of rabbits during the stage of vasodilatation. It will be noticed that

both arteries and veins show a definite narrowing of caliber on entry of the x-rayed area, and that the original caliber is largely if not entirely regained when these vessels issue from the x-rayed area.

DISCUSSION.

From the experimental facts described above and summarized in the table and charts, it is clearly evident that the skin of rabbits under certain conditions may acquire a remarkably increased resistance to doses of x-rays which are surely destructive to control animals. These conditions are that the animal whose skin tolerance to x-rays is to be increased must be sensitized with horse serum and this sensitization must take place before the rabbit is exposed to the x-rays.

The evidence for these conclusions is summarized in Text-figs. 1 and 2. In these charts it is shown that the standard dose of 30 skin units of x-rays causes a perforating gangrene of the ear in normal controls within 37 days after x-ray treatment. The same dose of x-rays administered to the ear of rabbits previously sensitized with horse serum (sensitized group in the chart) was, however, remarkably weakened in its effect upon the tissues exposed to the x-rays. Two animals showed no gangrene at all during the period of examination (over 340 days); one exhibited a perforating gangrene after the lapse of 131 days, and only one member of the group of four rabbits reacted fairly like the normal controls by developing a perforating gangrene in 46 days. That sensitization must be present before the animal is exposed to the standard test dose of x-rays, if protection from the ordinarily destructive effects of this dose is desired, is shown by the serum control group. These rabbits were normal animals and were injected with horse serum for the first time, but this injection took place 13 days after exposure to the x-rays. In this group all rabbits developed fenestration of the ears subsequent to dry gangrene within 50 days after exposure to the x-rays. The serum injection after x-ray treatment therefore conferred no marked trace of protection to the x-rayed areas of the ears.

Additional evidence to establish this point, that sensitization previous to x-ray treatment confers a marked increase in resistance, is furnished by the behavior of the x-rayed area in Rabbit HSX 13 of the sensitized group. In this animal the x-rayed area a number of times exhibited some crust formation with moderate congestion of the surrounding vessels. In addition, the internal surface presented a brownish, sunken discoloration such as frequently preceded the appearance of a perforating gangrene in the x-rayed areas of the control rabbits. Yet healing was fairly prompt and no perforating gangrene resulted. The recuperative power of this x-rayed area, therefore, was greater than that existing in the x-rayed areas of the controls.

Another observation which points to the same conclusion is the inflammatory reaction which occurred previous to fenestration within a portion of the x-rayed area of a sensitized rabbit, No. HSX 11. This inflammatory reaction was accompanied by a fair degree of redness and swelling and was much more pronounced than that observed in the x-rayed area of any other rabbit, though it was considerably less than the inflammation which the same rabbit showed in the adjoining untreated portion of the ear. This increased inflammatory response can only be interpreted as an expression of a more vigorous state of this x-rayed area when compared to that of rabbits of other groups.

Another fact which seems clear is that the protection to x-rays which sensitization previous to x-ray treatment gives, is largely abolished if these animals are reinjected with serum after being x-rayed; in other words, if they are subjected to an anaphylactic reaction.

The evidence for this statement is summarized in Text-figs. 1 and 2. The sensitized and reinjected group, it will be seen, was treated exactly like the sensitized group with but one exception: 13 days after being x-rayed and 23 days after the last sensitizing dose of serum, this group was reinjected with horse serum, and in consequence a mild, general anaphylactic reaction resulted, from which all promptly recovered. Nevertheless, the further course of the experiment showed that these reinjected rabbits had largely lost the protection which mere sensitization gives (see the sensitized group, Text-figs. 1 and 2), and dry gangrene with fenestration took place in due time. That some protection had still remained, however, is indicated by the fact that the interval between x-ray treatment and gangrene is appreciably longer in two animals (62 and 85 days respectively) than in any of the controls (see also Text-fig. 2).

The increased resistance of skin-covered tissues to unfiltered x-rays which results from previous sensitization with an undenatured protein may be roughly estimated from our data. In preliminary experiments we tested the effects of 15, 18, and $22\frac{2}{3}$ skin units of x-rays on areas of rabbits' ears 4 sq. cm. in size. With $22\frac{2}{3}$ units, perforating gangrene occurred in the two rabbits tested within 37 to 43 days. With 18 skin units, perforating gangrene took place 58 days after x-ray treatment in one rabbit, and incomplete gangrene in two others after 72 days, when observations were discontinued. With 15 skin units complete gangrene occurred in one rabbit after 91 days, incomplete gangrene in a second after 91 days, and no gangrene at all in a third animal after the same interval, when, unfortunately, all these rabbits were discarded.

From these incomplete data we may nevertheless conclude that sensitization with horse serum previous to x-ray treatment can reduce, at least in some animals, the destructive value of 30 skin units of x-rays to a level of 15 to 18 skin units.

It must not be forgotten that the conclusions which we have drawn so far rest upon experimental evidence gained under specific conditions which have been described in detail above. Further work must show whether modifications of these conditions entail significant changes in the result.

Our knowledge concerning the various factors involved is limited. We do not know fully what influence the degree of sensitization plays; whether or not a phase of increased susceptibility to the action of x-rays precedes the establishment of a heightened resistance; how long this increased resistance persists; what the maximum resistance is which can be attained by this procedure, and other questions.

To some of these questions a partial answer can be given. As far as the degree of sensitization is concerned, one may state that the rabbits employed were highly sensitized. In earlier series of experiments, the same sensitizing procedure, dose, and period of incubation had been used by one of us, and in these animals the intravenous reinjection test had yielded a high mortality rate. It must always be remembered, however, that the degree of sensitization which a certain fixed method achieves, fluctuates more widely in rabbits than in guinea pigs. This may explain why we failed to produce any sign of protection in one rabbit of the sensitized group (Text-figs. 1 and 2).

As far as the maximum amount of x-rays is concerned, our results with 30 skin units indicate that this dose is fairly close to the limit of tolerance with the experimental procedure employed.

Specificity.

The increased resistance to ordinarily lethal doses of x-rays which tissues may gain after a preliminary treatment with an undenatured foreign protein must be classed as a non-specific reaction, because the altered, abnormal response is called forth not by the sensitizing substance but by an utterly unrelated, physical agent. Such non-specific reactions after sensitization have been described and recognized for years. Heilner[7] in 1908 observed that serum-sensitized rabbits succumbed to an injection of 4 per cent sodium chloride which was practically harmless to normal controls. Davidsohn and

[7] Heilner, E., *Z. Biol.*, 1908, l, 487.

Friedemann[8] showed that rabbits sensitized with bovine serum react with temperature elevations to doses of sodium chloride given subcutaneously or intravenously, which produce no such effect in normal rabbits. Richet[9] noted that dogs sensitized by actino-congestine or crépito-congestine vomited after smaller doses of apomorphine hydrochloride, injected intraperitoneally, than normal dogs.

Non-specific reactions have also been utilized therapeutically,[10] but in this respect great caution is advisable. It must be realized that the incorporation of an undenatured foreign protein entails consequences of whose manifestations we are largely ignorant, and therefore often no intelligent balance can be struck between the harm and benefit which the procedure affords the patient. This deficiency in our knowledge should theoretically be lessened by laboratory work on the lower animals. For these reasons the irrational use of vaccines and sera is to be discouraged. Such powerful drugs should be used only when nothing else suffices to gain the desired therapeutic end. A conscious distinction should be drawn between drugs whose single injection exerts a comparatively short effect and those whose single injection releases reactions which are often masked and persist for months and even years. Sera and vaccines therefore may not be employed with the same careless freedom, which, for example, characterizes the use of various synthetic compounds.

Mechanism of Protection.

From the experimental data already presented and from the more obvious conclusions which we have so far drawn, no understanding of the underlying mechanism which brings about this increased resistance of the tissues to x-ray destruction can be reached. Some such

[8] Davidsohn, H., and Friedemann, U., *Berl. klin. Woch.*, 1909, xlvi, 1120; *Arch. Hyg.*, 1909, lxxi, 42.

[9] Richet, C., *Compt. rend. Soc. biol.*, 1910, lxviii, 820.

[10] For a review see Jobling, J. W., *The Harvey Lectures*, 1916–17, xii, 181; Miller, J. L., *J. Am. Med. Assn.*, 1921, lxxvi, 308; Cowie, D. M., *J. Am. Med. Assn.*, 1921, lxxvi, 310; Culver, H., *J. Am. Med. Assn.*, 1921, lxxvi, 311; Petersen, W. F., *J. Am. Med. Assn.*, 1921, lxxvi, 312.

basis can be obtained, however, if the results are considered in the light of a broad generalization of anaphylaxis. Such a generalization is the experimentally founded view[11] that an anaphylactic reaction is initiated when the anaphylactic antibody comes into contact with its antigen, during which process both antigen and antibody largely if not entirely disappear. If Text-figs. 1 and 2 are examined it will be observed that the only difference existing between the sensitized group and the sensitized-reinjected group is that the latter was subjected to an anaphylactic reaction 13 days after the x-ray treatment. In the sensitized-reinjected group, therefore, the anaphylactic antibodies had been removed more or less, while they were still present abundantly in the sensitized group. Since a majority of the sensitized group showed the marked resistance to massive doses of x-rays, while in the sensitized-reinjected group gangrene took place in the x-rayed area, it may be inferred that this protection is attributable to the anaphylactic antibodies which are present in the rabbits of the sensitized group, but which are not present, or at least not to the same functional degree, in the sensitized-reinjected group.

If the anaphylactic antibody is responsible for the protection to x-rays which the ears of the sensitized group exhibited, another inference may be drawn due to the fact that the x-ray treatment in the experiments was local. It follows that this protection must be assigned to the antibodies which are anchored to the tissue cells exposed to the x-rays and not to the circulating antibodies. This is shown clearly by the animals in the serum control group (Text-figs. 1 and 2). These animals had received one injection of horse serum, but this had been administered 13 days after the local x-raying of the ear. Within a short period an abundance of specific antibodies must have appeared in the circulation, and these necessarily must have traversed the capillary system of the x-rayed area of the ear. Yet practically no protection was conferred.[12] It appears, therefore, that the cells

[11] Anderson, J. F., and Frost, W. H., *J. Med. Research*, 1910, xxiii, 44; see also review by Wells, H. G., *Physiol. Rev.*, 1921, i, 51.

[12] A slight degree of protection is probably present. As shown in Text-fig. 1, the serum controls developed fenestration of the ears in 36 to 50 days while the normal controls attained the same state in a shorter time, 30 to 37 days. See other evidence in Text-fig. 2.

of an x-rayed area are unable to produce anaphylactic antibodies or to fix them, when present in the circulation, in sufficient amount to protect, provided that the x-ray treatment takes place before the injection of the antigen. The sensitized-reinjected group also supports this inference; in this group the antibodies formed as a result of the second injection of serum did not adequately replace those which were originally fixed in the x-rayed area but which were rendered inert by the anaphylactic reaction, though a certain measure of protection was observed (Text-figs. 1 and 2).

Finally, it may be inferred that the locally fixed anaphylactic antibodies (sensitized group) can be functionally removed by an anaphylactic reaction (sensitized-reinjected group) and the local protection which these fixed bodies gave against massive doses of x-rays is then largely abolished.[13]

It is probable that the increased resistance to x-rays conferred by a previous sensitization to the skin of rabbits will also be obtainable in man, and the procedure may thus be of utility in human therapeutics. Such a contingency will appear when malignant growths must be treated without the scalpel. Under these conditions the applicable dose of x-rays is directly limited by the resistance of the skin overlying the neoplasm for example, and a lethal dose for the cancerous tissue perhaps cannot be applied because it would also destroy the integument. This tentative proposal presupposes that the cancerous tissue does not acquire the same degree of resistance as the skin cells after sensitization, also that the heavy doses of x-rays do not ultimately produce malignant skin alterations. Concerning the first supposition, there is no knowledge available at present, but the experimental test is easily made; concerning the second, it may be said that no malignant changes in the skin of rabbits have been observed after a period of more than 300 days.[14] Finally, it may be stated that no objection can be urged against the parenteral employment of an undenatured foreign protein in such cases, because this effort is perhaps a last scientific attempt to help and it is therefore legitimate for the physician to invoke the aid of the protein molecule, fully conscious though he is that some or many of its various effects are not wholly desirable.

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[13] It is impossible to decide whether the moderate resistance of the sensitized-reinjected group is due to an imperfect removal of the anchored antibodies during the anaphylactic reaction or to anchorage of some antibodies subsequent to the anaphylactic reaction.

[14] This period of time in the rabbit is comparable to a much longer interval in the human subject, if we consider the relative length of life in the two species.

That sensitization with a foreign protein protects the skin from the harmful effects of a subsequent x-ray treatment is indicated by studies made by Hektoen. In a series of important observations[15] Hektoen studied the effect of massive doses of x-rays under various conditions upon the production of antibodies, the anaphylactic antibody not being included. His experimental material consisted of white rats, rabbits, and dogs, and the entire body of the animal was always subjected to the action of the x-rays. Hektoen established clearly that the time of x-raying with respect to the injection of antigen exerted a profound effect upon the antibodies. If the antigen was injected immediately after the preparatory x-ray treatment the production of antibodies was practically completely restrained. If, on the other hand, the x-raying was carried out at the height of antibody production (days or weeks after the antigen injection) no effect was noted on the antibody output.

The observations which interested us most, however, were as follows: When young puppies were x-rayed with strong doses of x-rays before they were injected with antigen (10 per cent rat or goat blood suspensions), severe burns of the skin were noted;[16] but if they were x-rayed about 7 days after the antigen injection, Hektoen apparently observed no burns, for he only states that now many dogs showed no disturbances of the general health.[17] If we are correct in this interpretation of Hektoen's work, our observations in this matter accord with his. We have not been able to find any other observations in the literature bearing upon this question.

Inflammation of the X-Rayed Area.

In the objective record of our results we have described three combinations in which inflammation of the x-rayed ear surface may appear. These three combinations, their distribution among the various experimental groups, and the duration of the process can be utilized to give further support to the antibody theory which has already been discussed.[18]

[15] Hektoen, L., *J. Infect. Dis.*, 1918, xxii, 28. This article gives the references to Hektoen's earlier work. See also Hektoen, L., *J. Infect. Dis.*, 1920, xxvii, 23.

[16] Hektoen, L., *J. Infect. Dis.*, 1918, xxii, 29.

[17] Hektoen,[16] p. 31.

[18] We have not included among the various types of inflammatory reaction the combination noted in Rabbit CX 18 (Table I). In this serum control rabbit the first inflammation exceptionally led at once to complete gangrene of a section of the x-rayed area. But 135 days later a subacute inflammation with crust formation developed and led to still another loss of tissue. This delayed second inflammation healed in 24 days (195 days after x-ray treatment). This type of reaction is probably allied to the delayed x-ray lesions which at times occur in the human subject months after the last treatment (Pfoerringer, S., Review in *Am. J. Roentgenol.*, 1917, iv, 642).

The three combinations of states are as follows:[19]

(1) First inflammation.....gangrene.
(2) First inflammation...healing.....second inflammation....gangrene.
(3) First inflammation.....healing.

The distribution of these combinations is summarized in Table I. An examination of this table shows that the second type or combination (first inflammation—healing—second inflammation—gangrene) occurs only in the groups which had been subjected at one time or another to the injection of horse serum. It was never observed in normal rabbits treated with a destructive dose of x-rays.

The first combination (first inflammation—gangrene) occurred in all of the three normal controls; it also was observed in two additional normal control animals which had been x-rayed with $22\frac{2}{3}$ skin units. But in the serum animals this combination was only noted two times (Rabbits CX 18 and HSX 8).

The third combination (first inflammation—healing) was only observed in the sensitized group, in which the horse serum was administered previous to x-ray treatment. It occurred two times out of four experiments, in Rabbits HSX 12 and HSX 13. A third rabbit of this group (No. HSX 11) shows a very marked prolongation in the interval between recovery from the first inflammation and the onset of the second inflammation which led to gangrene.

From the occurrence of the second type of combination (inflammation—healing—inflammation—gangrene) in eight out of twelve rabbits which had been treated with horse serum (Table I), and from the failure of this combination to appear in five normal control animals to which no serum had been given, we may infer that the increased resistance of the x-rayed tissue evinced by the second combination of states is definitely ascribable to the serum treatment. In other words, the administration of serum at any time within the limits employed in the experiments changes the reaction of the x-rayed tissue from the first combination (inflammation—gangrene) to the second combination (first inflammation—healing—second inflammation—gangrene) in the majority of the treated rabbits.

[19] It should be remembered that the first inflammation was a mild acute form, while the second inflammation was subacute in character.

We conclude, therefore, that this change was due to a protective antibody action which was produced by the parenterally injected horse serum. From the data given in this section no inference can be drawn concerning the type of antibody which caused this change of reaction to the standard dose of x-rays. Such an inference, however, can readily be drawn if we use the occurrence of gangrene and the duration of the entire process (Text-figs. 1 and 2) as criteria, and in a preceding section evidence has been presented that the anchored, anaphylactic antibody may be considered the protective factor. It is therefore probable that the same anchored anaphylactic antibody is also responsible for the altered character of the local reaction which the serum-treated rabbits exhibit after x-ray treatment. What part, if any, is played by other types of antibodies in this matter cannot be determined by the data at hand.

On the basis of these considerations the various successions of conditions observed in the x-rayed areas of the rabbits may be explained as follows: The inflammation observed in normal control animals which ends in gangrene is the normal slow, destructive action of our standard x-ray dose (30 skin units). The tissues exposed show a mild, acute inflammation which leads to a complete destruction of a portion of the x-rayed area. How these tissue changes are produced by the physical agent, the x-rays, we do not know;[20] vascular changes such as we have described undoubtedly are involved in the process.

If rabbits are treated with horse serum parenterally and exposed to the same standard x-ray dose, the type of reaction changes, due to the presence of anaphylactic reaction bodies anchored in the x-rayed area, the latter factor depending upon the time or times when the serum is administered. If the serum is administered about 2 weeks after x-ray treatment or if it is injected before and after x-ray treatment in such a way that a general anaphylactic reaction results, the second combination of conditions (inflammation—healing—inflammation—gangrene) then appears in the x-rayed areas of a majority of the rabbits (Table I, serum control group, sensitized-reinjected group).

[20] For a good presentation of the various theories concerning the mode of x-ray action upon tissues, see Hall, C. C., and Whipple, G. H., *Am. J. Med. Sc.*, 1919, clvii, 455.

The first inflammation now progresses to healing due to the presence of anaphylactic antibodies anchored in the x-rayed area. But this healing is only temporary, because the amount of locally available antibodies is too small or becomes functionally inert, and the slowly acting destructive forces gain the ascendancy over the reparative agencies. As a consequence the second inflammation appears which leads to a perforating gangrene. The second inflammation is subacute in character because the exposed area has been damaged by the x-rays, so that it can no longer react acutely to an inflammatory stimulus.

If, however, the serum is administered to a rabbit about 10 days previous to exposure to the standard dose of x-rays, the anaphylactic antibodies anchored in the x-rayed area may be sufficient in amount to protect that area for a long period or perhaps even indefinitely. The succession of conditions is then inflammation—healing—(Table I, sensitized group).

The explanation which we have given obviously only answers the question why the ordinary process of an x-ray action on tissues should be altered when the organism is treated with serum parenterally; how this alteration is produced we cannot say because it is not known how either the x-rays or the foreign protein exert their effects upon the tissue cells.

The results reported in this paper emphasize a precaution which ought to be observed in all animal experimentation. Since mere sensitization with an alien protein alters the reactivity of an organism not only towards the specific alien protein itself, but also towards an unknown number of other, unrelated substances or even physical agents, it is obvious that sensitized animals cannot serve as normal controls until it has been demonstrated that both the sensitized and normal animals react to the same agent in the same manner and to the same degree. Discarded animals which have been subjected experimentally to the action of undenatured proteins of bacterial, protozoan, metazoan, or vegetable origin should be used in identified groups when they are reemployed for an investigation. Failure to respect this precaution perhaps explains some of the discordant results obtained in diverse studies of the same problem. It is further possible that some of the erratic fluctuations in the degree of a reaction observed in a group of supposedly normal animals have their cause in an unsuspected proteinization of the abnormal reactors. The possibility or even probability of unwittingly employing proteinized mammalian material cannot be denied, for most investigators are compelled to rely upon dealers for their animal stock. In com-

pensation for this uncertainty, we may perhaps look upon the abnormal reactors among a group of animals as indicators of a possible proteinized state, and thus gain a working hypothesis which may add to our knowledge of non-specific phenomena.

SUMMARY.

When a fixed area of the ears of rabbits is subjected to the action of a standard destructive dose of x-rays (30 skin units) the type of reaction resulting depends upon the previous treatment of the rabbit. (1) In normal rabbits a mild acute inflammation develops in the x-rayed area which leads at once to a perforating gangrene within an average of 15 days. (2) If rabbits are x-rayed and about 2 weeks later injected with horse serum for the first time, a mild acute inflammation appears which heals for a time; then a second, subacute inflammation sets in which leads to a perforating gangrene. The average time of the process from the first inflammation to gangrene is 32 days. (3) If rabbits are sensitized with horse serum and 10 days later are exposed locally to the standard dose of x-rays, the ensuing ear reaction is either similar to the second reaction described above, except that it may last up to 110 days, or the first inflammation leads to a healing which may be apparently permanent (340 + days). (4) If rabbits are first sensitized with horse serum, exposed locally to the standard dose of x-rays 10 days later, and 13 days after the x-ray treatment reinjected with horse serum, the reaction of the x-rayed area of the ears is in general similar to the second reaction described above (inflammation—healing—inflammation—gangrene). The average time of the whole process is about 42 days.

On the basis of the general hypothesis that an anaphylactic reaction is initiated in the body when the specific antibody meets its antigen, and that both antibody and antigen are rendered more or less functionally inert by their interaction, the following inferences may be drawn from our experimental results. (1) The protection from the effects of a standard destructive dose of x-rays which a previous sensitization confers, is referable to the presence of anaphylactic antibodies in the x-rayed area. (2) This protection is largely due to the anaphylactic antibodies which are anchored in the x-rayed area, and not to those which are free in the circulation. (3) An anaphylactic reaction

renders the anchored anaphylactic antibodies largely impotent as protective factors against the standard destructive x-ray dose, even though sensitization preceded exposure to the x-rays. (4) An area treated with the standard destructive dose of x-rays is unable to produce or to anchor a sufficient amount of anaphylactic antibodies for protection from necrosis, when the x-ray treatment precedes the sensitization, or when the locally anchored anaphylactic antibodies are rendered functionally inactive by a general anaphylactic reaction.

It is possible that the procedure of increasing the resistance of the skin to a destructive dose of x-rays by means of a previous sensitization with protein may be applicable in the treatment of certain types of inoperable disease, when it is important to use massive doses of x-rays.

Animals which have been sensitized, or sensitized and reinjected with any undenatured alien protein, should not be reemployed as normal controls in any investigation unless trial has shown that these proteinized animals react quantitatively and qualitatively like normal animals.

The presence of an abnormal reactor in a group of supposedly normal animals may be an indication of a previous proteinization.

EXPLANATION OF PLATES.

The photographs of the rabbit ears were taken by transillumination while the blood vessels were in a dilated state. The time of photographing was 181 days after x-raying. The vessel traversing the middle of the ear is the central artery and in most figures its bifurcation near the upper pole of the ear can be seen. The marginal ear vein is also usually clearly visible. The x-rayed area of the right ear is shown as a bald quadrilateral space. Unfortunately the normal control rabbits were not photographed.

PLATE 100.

FIG. 1. Serum control group; Rabbit CX 16. Perforating gangrene occurred about 50 days after x-ray treatment.

FIG. 2. Serum control group; Rabbit CX 17. Perforating gangrene occurred about 50 days after x-ray treatment, but the process exceptionally is not yet complete although 181 days had passed since the x-raying. This is shown by the slowly healing, superficial ulcer to the right of the perforation, appearing as a black patch in the photograph.

PLATE 101.

FIG. 3. Sensitized group; Rabbit HSX 12. This figure shows that the bald, x-rayed surface is perfectly smooth with no perforation or crusts. The central artery in the x-rayed area is markedly narrowed. This area has remained in the same state for over 340 days after the date of x-ray treatment.

FIG. 4. Sensitized group; Rabbit HSX 13. The x-rayed area is intact and bald and the central artery shows clearly a partial stenosis. The small black spot represents a slight hemorrhage from a collection of fine, tortuous, superficial vessels at that point. The x-rayed area has remained in this condition now for more than 340 days.

PLATE 102.

FIG. 5. Sensitized and reinjected group; Rabbit HSX 5. The perforating gangrene took place 85 days after x-ray treatment. This figure shows clearly how both the central artery and the marginal ear vein are narrowed in the x-rayed field.

FIG. 6. Sensitized and reinjected group; Rabbit HSX 7. Perforating gangrene took place 62 days after x-ray treatment. The partial stenosis of a vein in the x-rayed area is shown to the right of the perforation.

FIG. 1.

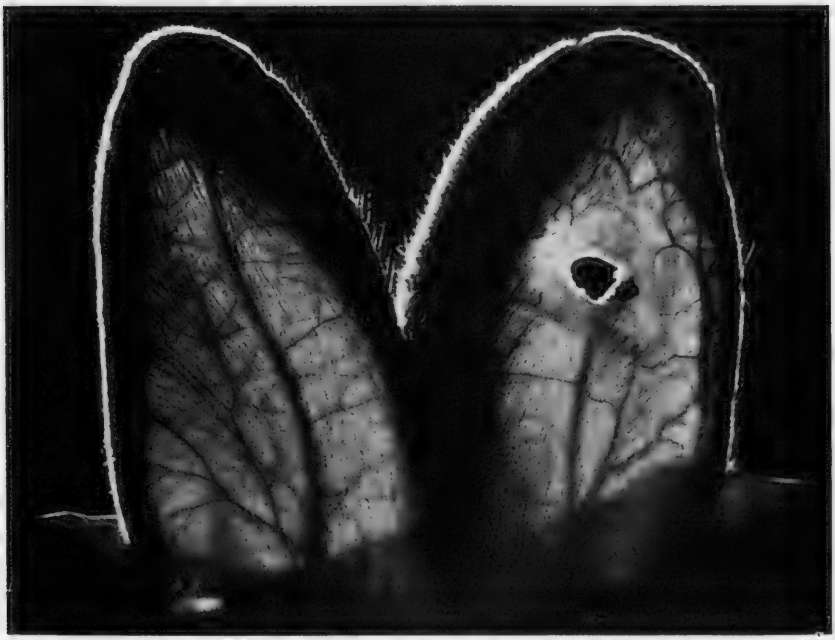

FIG. 2.

(Auer and Witherbee: Reaction of normal skin to x-rays.)

FIG. 3.

FIG. 4.

(Auer and Witherbee: Reaction of normal skin to x-rays.)

FIG. 5.

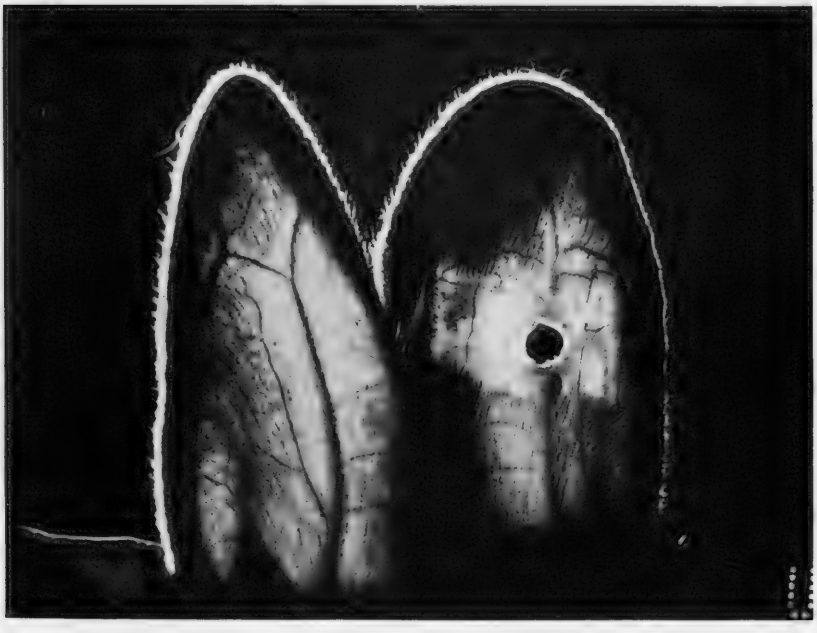

FIG. 6.

(Auer and Witherbee: Reaction of normal skin to x-rays.)

EFFECT OF SMALL DOSES OF X-RAYS ON HYPERTROPHIED TONSILS AND OTHER LYMPHOID STRUCTURES OF THE NASOPHARYNX.*

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Plates 103 to 108.

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In work carried on by us over several years we have extended the original observation of Heinicke[1] on the susceptibility of lymphoid tissue to x-rays and have shown other ways in which the x-rays may be employed as a therapeutic agent. It may be regarded now as established that the lymphoid tissue is more highly susceptible to x-rays than any of the structures of the body, except the sex glands, and that by suitable exposures it is possible to remove almost all of the lymphoid tissue without inducing detectable changes in other organs or tissues.[2] When the doses of x-rays are properly graded even the total number of polymorphonuclear leucocytes remains unaffected while the lymphocytes fall to a point at which few are seen in the circulating blood.[3]

The use of x-rays for reducing enlarged lymphoid organs is not new, but in the past the doses employed for the purpose have been large and thus have approached the danger point so closely that it has

* A preliminary report was made of this work before the American Society for Clinical Investigation and published in the Proceedings of the Society (Murphy, Jas. B., *J. Am. Med. Assn.*, 1920, lxxiv, 1738).

[1] Heinicke, H., *Mitt. Grenzgeb. Med. u. Chir.*, 1905, xiv, 21.

[2] Murphy, Jas. B., *J. Am. Med. Assn.*, 1914, lxii, 1459. Murphy, Jas. B., and Ellis, A. W. M., *J. Exp. Med.*, 1914, xx, 397.

[3] Taylor, H. D., Witherbee, W. D., and Murphy, Jas. B., *J. Exp. Med.*, 1919, xxix, 53.

been resorted to only in extreme instances. If, as our work indicates, extensive reduction of lymphoid tissue can be induced by small doses of x-rays, well within the safety limit, there is no reason why x-rays should not be used as a therapeutic agent in a variety of conditions. For obvious reasons the tonsil has been selected for the purpose of testing this point.

Histological examination of the tonsil shows it to have a structure similar to that of other lymphoid glands, with the exception of the fact that it is covered on one side by mucous membrane with crypts dipping down from the surface. These crypts have been described as natural test-tubes for the growth of bacteria. Whether or not the presence of pathogenic organisms in the crypts is the source of hypertrophy of the organ or whether the hypertrophy arises from another set of conditions is a moot point. It is, however, agreed that enlarged tonsils with resultant poorly drained crypts have a pathological significance.

In addition to the enlargement of the tonsil, other lymphoid deposits showing hypertrophy occur through the mucous membrane of the pillars of the fauces and as masses back of the posterior pillars. These structures also become pathologically altered in much the same way as the tonsil. They are not subject to surgical removal as is the tonsil but since they are made up of lymphoid cells are subject to influence by x-rays.

The following study was undertaken in order to test the effect of small doses of x-rays on the tonsil and other lymphoid deposits of the nasopharynx.

Technique.

The individuals to be treated are placed on a table with the head tilted so that the axis of the x-rays may pass under the angle of the jaw into the region of the tonsil. The area exposed on each side of the neck is about 3 inches square, the surrounding surface being covered with heavy sheet lead. The factors governing the dose of x-rays to each area were as follows: 8 inch spark-gap measured between points, 5 milliamperes, 10 inches distance from the target to the highest point of skin exposed; the time varies from 3 to 7 minutes, depending on the age of the individual, and the x-rays were filtered through 3 mm. of aluminum. The approximate value of this dose

is from one to one and three-quarters skin units. After an interval of a few weeks this treatment may if necessary be repeated with safety.[4] To insure immobility in young children a special board has been used with retaining straps and the child's head secured by means of a gauze bandage.

When excessive adenoid tissue was present a third area was exposed; namely, the back of the neck, just below the posterior occipital region with the head tilted forward. But this site of entry for the x-rays is less favorable, as will be indicated later in this paper.

Material.

This report is based on the study of 46 individuals ranging in age from $3\frac{1}{2}$ to 45 years and observed 1 month or longer after treatment. About 40 other individuals were treated, but as they did not return for examination they have not been included. The condition of the tonsils was noted in each individual and a drawing showing the size made by an artist independent of the examining physician. The state of the tonsils on first examination varied from that of simple hypertrophy to the enlarged organ with ragged surface and deep crypts containing exudate, or the small pathologically altered tonsil associated with symptoms of systemic disease. No individuals were treated at a time when the throat showed signs of acute inflammation.

The histories of eleven selected typical cases will be given and drawings of some of these, showing the progressive changes in the throat.

Case A.—J. L. L., white, male; age 19 years (Fig. 1).

Mar. 15, 1920. Throat: The tonsils, small and buried, the left larger than the right, ragged in appearance; numerous crypts containing exudate. Lymphoid

[4] In a later study Witherbee has recommended the use of fractional doses, four or more if necessary, treatments being given at 2 week intervals (Witherbee, W. D., *Am. J. Roentgenol.*, 1921, viii, 25). The factors of this dose are, 7 inch spark-gap measured between points, 5 milliamperes, 10 inches distance, time of exposure 3 minutes and 18 seconds; filtered through 3 mm. of aluminum. The principal advantage of this method is that it makes the treatment more flexible and the individual may be given more nearly the amount of x-rays necessary to induce the desired result.

tissue behind pillars increased in amount; large amount of adenoid tissue. Enlarged cervical lymph nodes. Bacteriological examination:[5] Right tonsil 50, left tonsil 150 colonies of hemolytic streptococci; vault none. X-rays: Three areas, right and left sides of neck and posterior occipital region, exposed to filtered x-rays; spark-gap 8 inches, milliamperes 5, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 22. Tonsil tissue markedly reduced and glazed and pale in appearance; lymphoid tissue behind pillars pale and smooth. Bacteriological examination: Right tonsil 50, left 150, and vault 50 colonies of hemolytic streptococci.

Apr. 5. Tonsil pale; edges of crypts inverted; no exudate can be pressed from tonsil. Adenoids considerably reduced; smooth and pale. Bacteriological examination: Right tonsil 100, left tonsil 50, and vault 150 colonies of hemolytic streptococci.

Apr. 26. Little visible tonsil tissue, of smooth appearance; no exudate; adenoid tissue small in amount, smooth and regular. Bacteriological examination: No hemolytic streptococci found.

Case B.—E. S., white, male; age 26 years (Fig. 2).

Dec. 10, 1919. Throat: Medium sized tonsils with deep crypts full of exudate; ragged inflamed surface. X-rays: Two areas, right and left side of neck, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 4, 1920. Tonsil shows marked shrinkage; some exudate present. X-rays: Two areas, right and left side of neck, exposed to x-rays in same dosage as above.

Apr. 4. Tonsils small; smooth surface; no exudate.

Mar. 5, 1921. Tonsils small with smooth surface; no exudate.

Case C.—H. W., white, male; age 14 years.

Mar. 17, 1920. Throat: Tonsils very large, buried; irregular surface; numerous crypts with yellowish exudate. Mass of lymphoid tissue back of pillars. Adenoid tissue, large mass covering surface of vault and fossa. Bacteriological examination: Right tonsil no colonies, left 50, and vault 50 colonies of hemolytic streptococci. X-rays: Three areas, right and left side of neck and posterior occipital region, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 24. Tonsils show decided reduction in size; pale, smooth surface; on pressure some whitish secretion. Adenoid tissue considerably reduced and of smooth, clean appearance. Bacteriological examination: Tonsils no colonies, vault 50 colonies of hemolytic streptococci.

Mar. 31. Tonsils show further reduction in size; smooth and pale; no exudate on pressure. Adenoid tissue pale, smooth, and clean; more normal in appearance. Bacteriological examination: Tonsils and vault show no hemolytic organisms.

Apr. 7. Improvement continues. Lymphoid tissue along posterior pillars has entirely disappeared.

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[5] It is obvious that the number of colonies is roughly indicative only, as the quantity of material inoculated and part of organ touched with the loop are not subject to accurate control.

June 14. Tonsils flat; pale smooth surface; adjacent mucous membrane pale; edges of crypts inverted and crypts show retraction. Adenoid mass materially reduced. Bacteriological examination: No cultures taken.

Case D.—S. A., white, male; age 31 years.

Apr. 7, 1920. Throat: Tonsils moderately enlarged, buried; numerous crypts; purulent fluid on pressure. Marked hypertrophy of lymphoid tissue along posterior pillars. No adenoid tissue. Bacteriological examination: Right tonsil 200, left tonsil 200, and vault 200 colonies of hemolytic streptococci. X-rays: Three areas, right and left side of neck and posterior occipital region, exposed to x-rays. Spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Apr. 12. Tonsils show some reduction; pale and smooth; very little exudate on pressure. Marked reduction in lymphoid tissue on posterior pillars. Bacteriological examination: Right tonsil 150, left tonsil 200, and vault no colonies of hemolytic streptococci.

Apr. 19. Tonsils markedly reduced, smooth, pale, normal in appearance; less exudate on deep pressure. Further reduction in amount of lymphoid tissue on pillars. Bacteriological examination: Right tonsil 50 colonies, left tonsil no colonies, and vault 50 colonies of hemolytic streptococci.

May 10. Tonsils further reduced; mucous membrane of tonsil and pillars smooth and pale; edges of crypts rounded; small amount of exudate on deep pressure. Lymphoid deposit on pillars has practically disappeared. Bacteriological examination: No hemolytic streptococci found.

Case E.—J. V. K., white, male; age 15 years (Fig. 3).

Mar. 31, 1920. Throat: Tonsils large with ragged, irregular surface; numerous crypts with exudate. Large irregular mass of adenoid tissue. Bacteriological examination: Right tonsil 150 colonies of hemolytic staphylococci, 50 colonies of hemolytic streptococci; left tonsil 50 colonies of hemolytic streptococci; vault 100 colonies of hemolytic staphylococci. X-rays: Three areas, right and left side of neck and posterior occipital region, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Apr. 7. Tonsils slightly reduced; less ragged in appearance. Adenoids show some reduction. Vault contains mucopurulent discharge. Bacteriological examination: Right tonsil 100, left 50, and vault no colonies of hemolytic streptococci.

Apr. 14. Tonsils markedly reduced; surface irregular, ragged, and congested. Adenoid tissue reduced; smoother surface. Bacteriological examination: No hemolytic organisms found.

Apr. 21. Tonsils show still further reduction; surface smooth; edges of crypts inverted. Adenoid tissue markedly reduced; smooth pale surface. Bacteriological examination: No hemolytic organisms found.

Apr. 26. Tonsils flat; pale, smooth, clean surface; edges of crypts round and inverted. Bacteriological examination: No hemolytic organisms found.

May 24. Tonsils normal in appearance. Adenoid mass reduced in size.

Sept. 29. Tonsils very small; smooth, pale surface. Adenoids greatly reduced and normal in appearance.

Case F.—S. V. M., white, male; age 10 years.

Mar. 24, 1920. Throat: Tonsils large, partly buried; irregular, ragged surface; numerous crypts filled with thick yellow exudate. Lymphoid tissue behind pillars markedly hypertrophied. Large irregular mass of adenoid tissue covered with purulent exudate. Bacteriological examination: Right tonsil 200, left tonsil 100, and vault no colonies of hemolytic streptococci. X-rays: Three areas, right and left side of neck and posterior occipital region, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 31. Tonsils reduced in size; edges of crypts inverted; less exudate. Pharynx had dull red glazed appearance. Adenoid tissue considerably reduced; pale; less exudate. Bacteriological examination: Right tonsil 50 colonies of hemolytic streptococci, left tonsil and vault no hemolytic organisms.

Apr. 7. Tonsils markedly reduced; some surface secretion; crypts much cleaner. Adenoid tissue still further reduced. Bacteriological examination: No hemolytic organisms found.

Apr. 14. Tonsils very markedly reduced; surface smooth and clean; edges of crypts inverted and smooth; no exudate on deep pressure. Adenoid tissue smooth; normal appearance. Lymphoid tissue back of posterior pillars practically disappeared. Bacteriological examination: No hemolytic organisms found.

Sept. 29. Tonsils show further reduction; normal in appearance; edges of crypts smooth and inverted. Adenoid tissue still present; small amount of exudate on pressure.

Case G.—J. W., white, male; age 17 years (Fig. 4).

Mar. 24, 1920. Throat: Tonsils very large; ragged and congested. Considerable hypertrophy of lymphoid tissue behind posterior pillars. Bacteriological examination: Right tonsil 200, left tonsil 150, and vault 200 colonies of hemolytic streptococci. X-rays: Three areas, right and left side of neck and posterior occipital region, x-rayed; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 31. Tonsils showed marked reduction; pale and smooth. Bacteriological examination: Right tonsil 100, left tonsil 50, vault 100 colonies of hemolytic streptococci.

Apr. 14. Tonsils show still further reduction; smooth, normal appearance. Lymphoid tissue behind posterior pillars entirely gone. Bacteriological examination: No hemolytic organisms found.

June 28. Tonsils small, smooth, pale; edges of crypts inverted and translucent.

Sept. 13. Tonsils show some further reduction; white bands around edges of inverted crypts; right tonsil shows some cheesy deposits, easily removed on pressure. Bacteriological examination: Right tonsil 50 colonies of hemolytic streptococci, left tonsil and vault no hemolytic organisms.

Feb. 17, 1921. Tonsils small, flat; surface smooth and pale; no exudate. Bacteriological examination: No hemolytic organisms found.

Case H.—J. Z., white, male; age 21 years (Fig. 5).

Mar. 10, 1920. Throat: Tonsils moderately enlarged; ragged surface; numerous crypts. Left tonsil has large fossa filled with purulent exudate. Moderate sized mass of adenoid tissue, irregular, covered with whitish secretion. Bacteriological examination: Right tonsil no hemolytic organisms; left tonsil 50 colonies of hemolytic streptococci and 200 colonies of hemolytic staphylococci; vault 50 colonies of hemolytic streptococci and 50 colonies of hemolytic staphylococci. X-rays: Three areas, right and left side of neck and posterior occipital region, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 17. Tonsils distinctly reduced; cleaner in appearance. Adenoids cleaner. Bacteriological examination: No hemolytic organisms found.

Mar. 31. Tonsils reduced; still some exudate. Adenoid tissue reduced, pale; less exudate.

Apr. 28. Tonsils show marked reduction; smooth, pale surface; crypts clean; no exudate on deep pressure.

June 14. Tonsils show still further reduction; no exudate from right tonsil, small amount from left on deep pressure. Adenoids possibly reduced.

Sept. 13. Tonsils small, smooth, pale; on deep pressure still some exudate; edges of crypts markedly inverted. Adenoid tissue still present.

Mar. 11, 1921. Tonsils small; normal in appearance.

Case I.—V. S., white, male; age 14 years (Fig. 6).

Mar. 3, 1920. Throat: Tonsils large, ragged; numerous crypts full of pus. Large mass of lymphoid tissue behind posterior pillars. Large mass of adenoid tissue. Bacteriological examination: Right tonsil 100 colonies of hemolytic streptococci and 50 colonies of hemolytic staphylococci; left tonsil 50 each of hemolytic streptococci and staphylococci; vault 50 colonies of hemolytic streptococci and 150 of hemolytic staphylococci. X-rays: Two areas, right and left side of neck, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 7 minutes, filtered through 3 mm. of aluminum.

Mar. 10. Tonsils reduced in size; smooth surface; less exudate on pressure. Lymphoid masses behind posterior pillars markedly reduced; smooth glazed appearance. Bacteriological examination: Right tonsil 50 colonies of hemolytic streptococci, left tonsil and vault no hemolytic organisms found.

Mar. 17. Tonsils still further reduced; pale, clean. Bacteriological examination: No hemolytic organisms found.

Mar. 24. Tonsils smaller; smooth surface. Further reduction in lymphoid tissue back of pillars. Bacteriological examination: No hemolytic organisms found.

Apr. 28. Tonsils show marked reduction in size; pale and smooth; no injection of mucous surfaces; no exudate on deep pressure; edges of crypts smooth. Lymphoid tissue behind posterior pillars practically disappeared. Adenoid mass reduced. Bacteriological examination: No hemolytic organisms found.

Sept. 13. Tonsils small and normal in appearance; no exudate on deep pressure; edges of crypts smooth and inverted; mucous surfaces show no injection. Lymphoid deposits back of pillars practically gone. Adenoids reduced but still large.

Case J.—E. C., white, male; age 16 years.

Mar. 22, 1920. Throat: Tonsils large; numerous crypts filled with yellowish exudate; large amount of cheesy material on pressure. Hypertrophy of lymphoid tissue on posterior pillars. Large irregular mass of adenoid tissue covered with yellowish exudate. Bacteriological examination: Right tonsil 200, left 150, and vault 150 colonies of hemolytic streptococci. X-rays: Two areas, right and left side of neck, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 29. Tonsils considerably reduced; smooth and pale; no exudate on pressure. Adenoid tissue considerably reduced. Bacteriological examination: Right tonsil 200, left 150, and vault 150 colonies of hemolytic streptococci.

Apr. 5. Tonsils further reduced; pale and smooth; edges of crypts smooth; slight exudate on pressure. Adenoids markedly reduced; pale, smooth, and clean. Bacteriological examination: Right tonsil 50, left 50, and vault no colonies of hemolytic streptococci.

Apr. 14. Tonsils and adenoids markedly reduced; pale and smooth. Bacteriological examination: No hemolytic organisms found.

May 10. Tonsils small; normal in appearance; mucous surfaces pale; edges of crypts rounded and smooth; no exudate on deep pressure. Lymphoid tissue on posterior pillars has practically disappeared. Adenoid mass considerably reduced in size.

June 14. Tonsils normal in appearance. Adenoids very small, pale, smooth mass.

Sept. 29. Tonsils and adenoids small and normal in appearance; surface smooth; no exudate.

Case K.—J. F., white, male; age 16 years.

Mar. 8, 1920. Throat: Tonsils large, ragged; numerous crypts. Large mass of adenoid tissue. Enlargement of cervical glands. Bacteriological examination: Right tonsil 100, and left tonsil 50 colonies of hemolytic streptococci. X-rays: Two areas, right and left side of neck, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 7 minutes, filtered through 3 mm. of aluminum.

Mar. 15. Tonsils reduced; smooth, glazed surface. Adenoids considerably reduced; smooth and glazed. Bacteriological examination: Right tonsil 100, left tonsil 150, and vault no colonies of hemolytic streptococci.

Mar. 29. Tonsils further reduced; pale and smooth. Adenoids also reduced; pale and smooth. Bacteriological examination: No hemolytic organisms found.

Apr. 5. Right tonsil more reduced than left; surface pale and smooth. Adenoid mass pale, clean.

Apr. 12. Both tonsils further reduced; crypts inverted. Adenoid tissue considerably reduced. Bacteriological examination: Right tonsil 50 colonies of hemolytic streptococci, left tonsil and vault no hemolytic organisms.

May 10. Tonsils markedly reduced; surface normal; edges of crypts rounded and inverted; on deep pressure still slight amount of exudate. Lymphoid tissue behind posterior pillars completely disappeared. Adenoid tissue reduced but still large. Enlarged cervical glands somewhat smaller. Bacteriological examination: Right tonsil 5 colonies of hemolytic streptococci, left tonsil and vault no hemolytic organisms.

Sept. 29. Tonsils markedly reduced; normal in appearance; edges of crypts inverted, pale; no exudate. Adenoid tissue still present.

Table I shows the class of cases which have been treated with x-rays and the result of this treatment.

In general it may be said that the reduction in size of the tonsils and other lymphoid deposits in and behind the pillars of the fauces becomes clearly evident about 2 weeks after treatment and increases for 1 to 2 months. As the tonsil shrinks the crypts open and drain, and finally the edges become inverted and the surface grows smooth and healthy in appearance. The small lymphoid deposits in the pillars and the larger accumulation frequently present behind the posterior pillars of the fauces disappear promptly after the treatment and at the same time the injection of the vessels subsides.

The adenoid tissue is, as expected in view of the portal of entry used for the x-rays, not so uniformly reduced as the tonsils. This particular aspect of the problem of reducing excessive lymphoid tissue in the nasopharynx through x-rays is one to which in the near future especial attention will need to be given.

Bacteriological Examination.

Cultures were taken from the throats of 40 of the 46 individuals before and at intervals after treatment. The material for culturing was obtained from the crypts of the right and left tonsils, by means of a platinum wire about 3 inches long at the end of which was a small loop 2 mm. in diameter, bent at a right angle to the main piece of wire. The tongue was pressed down firmly with a tongue depressor, and the wire introduced into the crypt of the tonsil with a "stab and twist" motion. Caution was used to avoid contamination from

TABLE I.

Individual No.	Age.	Sex.	Tonsils.					Adenoids.	
			Size before treatment.	Condition.	Length of time under observation after treatment.	Size at last examination.	Condition after treatment.	Size and condition before treatment.	Size and condition after treatment.
	yrs.				*mos.*				
1	31	M.	+++*	Numerous crypts; mucopurulent exudate; considerable lymphoid tissue behind pillars.	2	++	Mucous membrane smooth; lymphoid tissue behind pillars almost gone; general appearance normal.	None.	
2	35	M.	+++	Soft, friable; numerous crypts; thin purulent exudate.	1	+	Edge of crypts smooth; normal.	Small.	Some reduction.
3	13	M.	++++	Ragged, inflamed; exudate.	6	++	Smooth, pale; right tonsil still not normal.	Large.	Little affected by treatment.
4	29	M.	++++	Ragged; deep crypts.	4	++	Crypts smooth and shallow; normal appearance.	Large.	Considerable reduction.
5	25	F.	++++	Not markedly abnormal.	3	++	Edges of crypt inverted; normal.	Small.	Reduction.
6	16	M.	++++	Numerous crypts; yellow, cheesy exudate; large mass of lymphoid tissue posterior to pillars.	6	++	Surface normal; no exudate in crypts; lymphoid masses posterior to pillars gone.	Large; covered with exudate.	Marked reduction; normal surface.

7	7	M.	+++++	Ragged; crypts contain mucopurulent exudate.	5	+++	Improvement; tonsils still not normal.		
8	15	F.	+++++	Numerous crypts with purulent exudate.	1½ *wks.*	+++	Normal appearance; crypts inverted.	Large.	Reduction.
9	30	F.	+++	Ragged, inflamed; numerous crypts; exudate.	3 *mos.*	++	Clear, normal.	Medium.	Reduction.
10	?	M.	+++	Deep crypts with cheesy exudate; mass behind pillars.	5	++	Some exudate on deep pressure; surface normal; posterior pillar deposits absent.	Medium.	Reduction.
11	7	F.	+++	Ragged; deposits posterior to pillars.	5	++	Smooth; normal appearance; deposits posterior to pillars gone.	Large.	Marked reduction.
12	6½	F.	++++	Numerous crypts with ragged edges.	5	+	Normal.	Small.	No reduction.
13	15	M.	++++	Ragged, soft, congested.	1	++	Absolutely normal appearance.	Medium.	Marked reduction.
14	16	M.	++++	Numerous crypts; ragged edges; deposit behind pillars.	6½	++	Normal appearance; deposits behind pillars gone.	Large.	No marked reduction.
15	3	M.	++++	Ragged.	1	++	Much improved.	Very large.	Reduction.
16	6	F.	++++	Congested; numerous crypts; ragged; deposits posterior to pillars very large.	5½	++	Absolutely normal; deposit back of pillars gone.	Medium.	No marked reduction.

* +++++ very much enlarged; ++++ much enlarged; +++ medium enlargement; ++ approximately normal; + small; − not visible.

TABLE I—*Continued.*

Individual No.	Age.	Sex.	Tonsils.					Adenoids.	
			Size before treatment.	Condition.	Length of time under observation after treatment.	Size at last examination.	Condition after treatment.	Size and condition before treatment.	Size and condition after treatment.
	yrs.				*mos.*				
17	5	M.	++++	Ragged.	1½	+++	Edges of crypts inverted; smooth surface.	Very large.	No marked reduction.
18	49	M.	++++	Ragged; large deposit back of pillars.	6	++	Normal; posterior pillar deposits greatly reduced.	Large; ragged.	Some reduction; normal appearance.
19	28	F.	++++	Yellow, cheesy exudate; numerous crypts.	2½	+	Normal.	Medium.	Definite reduction.
20	28	F.	++	Ragged; numerous large crypts.	1	+	Normal; surface smoothed out.	Medium; mucopurulent exudate.	Reduction; no exudate.
21	15	M.	++++	Ragged; large crypts.	6	+	Normal; inversion of crypt.	Large; irregular.	Reduction; smooth.
22	35	M.	+++	Inflamed; ragged; numerous crypts; exudate.	1	++	Normal appearance; smooth, clean surface.		
23	7	F.	+++	Ragged.	2	+	Normal appearance.	Large, irregular.	Some reduction.
24	6	F.	++++	Ragged; numerous crypts; mucopurulent exudate.	2	++	Smooth, normal surface.		
25	26	M.	+++	Cheesy exudate.	3	+	Perfectly normal.	Small.	Reduction.

26	19	M.	+	Ragged; numerous crypts.	1	−	Normal appearance; crypts free from exudate.	Large.	Great reduction.
27	10	M.	++++	Ragged; purulent exudate in crypts.	6	+	Normal clean surface; crypts inverted.	Large.	Slight reduction.
28	7	M.	+++	Numerous crypts; pale and ragged.	2	++	Normal; crypts inverted.	Large.	No marked reduction.
29	33	M.	+++	Purulent exudate; crypts irregular with cheesy deposit.	10	+	Normal.	Medium.	Reduction.
30	6	F.	++++	Irregular ragged surface; mucous membrane injected.	2	++	Clean; mucous membrane pale.	Large.	No marked reduction.
31	4	F.	+++	Ragged, congested.	2	++	Surface clear.†	None.	
32	17	M.	+++	Irregular surface, congested; thick yellow exudate; deposits posterior to pillars.	6	++	Surface pale and clean; normal.	Small.	Reduction.
33	35	M.	+++	Yellow exudate in crypts; lymphoid tissue back of pillars.	1½	+	Normal appearance.		
34	45	F.	+++	Cheesy exudate.	5	+	Surface clear; one tonsil still has some exudate on deep pressure.		
35	31	M.	++++	Ragged, congested; purulent exudate and cheesy deposits.	1	++	Normal appearance.	None.	
36	14	M.	++++	Crypts contain pus; lymphoid deposits back of pillars.	6	+	Normal appearance; deposits back of pillars reduced.	Large.	Reduction.

†Later had an attack of tonsillitis.

TABLE I—*Concluded.*

Individual No.	Age.	Sex.	Tonsils.					Adenoids.	
			Size before treatment.	Condition.	Length of time under observation after treatment	Size at last examination.	Condition after treatment.	Size and condition before treatment.	Size and condition after treatment.
	yrs.				*mos.*				
37	36	F.	++	Mucopurulent exudate; lymphoid tissue back of pillars.	5	+	Normal.	Small.	No marked reduction.
38	17	M.	++++	Ragged, congested; lymphoid deposit back of pillars.	8	+	Smooth, pale; some exudate on deep pressure; lymphoid deposits gone.	None.	
39	21	F.	+++	Ragged, congested.	3	+	Normal.	None.	
40	30	F.	++++	Not abnormal.	1	+	Normal.	Small.	Little reduction.
41	23	F.	++	Mucopurulent exudate; cheesy deposits.	5	+	Smooth surface, normal appearance.	None.	
42	14	F.	++	Mucopurulent exudate; cheesy deposits; soft and friable.	5	+	Surface pale and normal; some exudate on deep pressure.	Large.	Little reduction.
43	14	M.	++++	Yellowish exudate.	3	+	Normal.	Large.	Reduction.
44	21	M.	++	Cheesy exudate.	1	+	Normal appearance; still some exudate on deep pressure.	Small.	Little reduction.
45	21	M.	++++	Subacute inflammatory condition.	8	+	Normal appearance; small amount of exudate on deep pressure.	Small.	Little reduction.
46	25	M.	+++	Inflamed; covered with mucopurulent exudate.	14	+	Absolutely normal.	None.	

the tonsil surfaces and the saliva. The material, so obtained, was placed on a blood agar plate, and gently spread over the surface with another platinum wire. The plates were then placed in the incubator at 37.5°C. Examinations of the plates and records of the findings were made after 24 and 48 hours of incubation.

Cultures were also taken from the nasopharyngeal vault by means of a very thin, semicircular, platinum wire, at the end of which was a small loop similar to that of the wire used in culturing the crypts of the tonsils. After firmly depressing the tongue, this wire was introduced into the vault directly back of the nose. The examination of the blood agar plates of these cultures was also made after incubating for 24 and 48 hours.

No attempt was made to differentiate the more common organisms usually found in the throat, such as pneumococcus, *Streptococcus viridans*, *Staphylococcus albus*, and *Staphylococcus aureus*. In distinguishing between hemolytic streptococcus and hemolytic staphylococcus, subcultures and stained films were resorted to. When the colony ranged in size from a pin-point to a small pin-head, and the area of hemolysis around the colony was from three to five times greater, streptococcus was usually found. Colonies which were as large as pin-heads or greater in diameter and about which the zone of hemolysis was very slight, appearing as a small halo, were usually staphylococcus.

36 of the 40 individuals showed hemolytic organisms to be present. Of these, seven became negative 1 week after treatment, fourteen after 2 weeks, eight after 3 weeks, and one after 4 weeks, making a total of 30 of the 36 treated cases which became negative. The six which continued to show hemolytic organisms were lost track of before further observations could be made.

Blood Counts.

The results of the x-ray treatment on the white blood cell count were not uniform. The counts were made at irregular intervals after meals and after a walk of several city blocks. When the lymphocytes were reduced in numbers the reduction was slight and of short duration, which would indicate that the systemic effect of the dose used was very slight.

DISCUSSION.

The small series of cases reported here shows the possibility of materially reducing the lymphoid deposits of the nasopharynx by comparatively small doses of x-rays. Animal experiments had shown that it is possible with x-rays to induce any degree of atrophy of the lymphoid tissue without damaging other tissues. In the series of treated individuals, in all but three or four instances one treatment gave an entirely satisfactory result. In two refractory cases a second treatment was followed by the desired degree of atrophy and a clearing up of the pathological condition. It is most probable that the other few individuals who did not respond to the one treatment would have yielded on further exposure to x-rays but unfortunately the observations were discontinued before this point could be determined.

The degree of atrophy to be aimed at is a matter that experience will decide. If a reduction below the normal size and the clearing up of obvious pathological states is sufficient as has been indicated in the majority of the cases treated and observed by us, there seems to be no reason for carrying the treatment beyond this point. In view, however, of the mild nature of the treatment recommended it appears entirely safe to repeat it at suitable intervals so as to secure almost any degree of atrophy that may be desired.

Our original idea in taking up this work was that the excess of lymphoid tissue interfered with the clearing up of local infections of the pharynx. It seems probable, however, that the disappearance of infection of the tonsils and change in bacteriological flora after x-ray treatment are due to the opening up and proper drainage of the crypts which follow atrophy, rather than the actual removal of the excess lymphoid tissue.

Tonsils which have been exposed to the x-rays and not sufficiently reduced in size would in all probability be as amenable to surgical removal as before the x-ray treatment, for we have never seen any evidence of fibrosis in the lymphoid organs of animals after similar treatment. The fibroid tonsil would probably not be reduced materially by x-rays, as fibrous tissue is not appreciably affected by this agent.

SUMMARY.

46 individuals with tonsils both hypertrophied and otherwise pathologically altered and some of whom had in addition adenoid masses and lymphoid deposits posterior to the pillars of the fauces, were given exposures to x-rays. In all but four cases the treatment was followed by marked atrophy of the tonsils and the other lymphoid deposits, attended by an opening and drainage of the tonsillar crypts. As this process progressed the previously enlarged tonsils assumed a smooth and normal appearance and the hemolytic bacteria—streptococci and staphylococci chiefly—which were often present in the affected tonsil disappeared usually within 4 weeks of the treatment.

EXPLANATION OF PLATES.

PLATE 103.

FIG. 1, *a* to *d*. (*a*) Condition of the tonsils before treatment; small and partly buried, with ragged surface and crypts containing exudate. In addition there were deposits behind the posterior pillars. (*b*) 1 week after x-ray treatment, showing reduction, and smoothing out of surface. (*c*) 3 weeks after treatment, with further reduction; no exudate could be expressed from tonsils. (*d*) 6 weeks after treatment. Tonsils not visible till anterior pillar was pulled back; normal in appearance. Hemolytic streptococci were present at the first examination but had disappeared by the 6th week after treatment.

PLATE 104.

FIG. 2, *a* to *f*. (*a*) The tonsils before treatment; medium size, with deep crypts containing exudate; ragged, inflamed surface. (*b*) 8 days after treatment; some reduction. (*c*) 26 days after treatment; tonsils congested and exudate still present. (*d*) 55 days after treatment; tonsils reduced but not normal. (*e*) 1 week after a second x-ray treatment, showing further reduction, and smoothing out of surface. (*f*) 1 year and 1 month after second treatment; tonsils small and normal in appearance.

PLATE 105.

FIG. 3, *a* to *d*. (*a*) Condition of tonsils before treatment; ragged, irregular surface, with crypts containing exudate. (*b*) 2 weeks after x-ray treatment; tonsils markedly reduced; surface irregular and congested. (*c*) 7 weeks and 5 days after treatment; tonsils normal in appearance. (*d*) 6 months after treatment; tonsils very small and normal. Hemolytic streptococci, present on first examination, disappeared after treatment.

PLATE 106.

FIG. 4, *a* to *e*. (*a*) Tonsils before treatment; very large, ragged, and congested. Hypertrophy of lymphoid tissue behind posterior pillars. (*b*) 3 weeks after treatment; tonsils reduced in size. Lymphoid tissue behind posterior pillars entirely atrophied. (*c*) 2 months after treatment; tonsils further reduced; normal in appearance. (*d*) About 6 months after treatment; tonsils further reduced; normal surface; still some exudate on deep pressure. (*e*) 11 months after treatment; tonsils small, flat; no exudate. Hemolytic streptococci disappeared from the throat after treatment.

PLATE 107.

FIG. 5, *a* to *e*. (*a*) Condition of tonsils before x-ray treatment; enlarged; ragged surface; deep crypts, with purulent exudate. (*b*) 7 weeks after treatment; tonsils markedly reduced; smooth, pale; no exudate on deep pressure. (*c*) 14 weeks after treatment; tonsils still further reduced; surface normal; no exudate. (*d*) 6 months after treatment; tonsils small and normal. (*e*) 1 year and 1 month after treatment; tonsils small and normal. Hemolytic streptococci disappeared from the throat by 1st week after treatment.

PLATE 108.

FIG. 6, *a* to *e*. (*a*) Tonsils before x-ray treatment; large, ragged; crypts contain pus. Large mass of lymphoid tissue behind posterior pillars. (*b*) 2 weeks after treatment; tonsils reduced; surface smooth and clean. Mass behind pillars reduced. (*c*) 4 weeks after treatment; tonsils markedly reduced; pale and smooth; no exudate on deep pressure. (*d*) 8 weeks after treatment; small amount of exudate. (*e*) 6 months after treatment; tonsils small, normal in appearance; no exudate on deep pressure. Lymphoid tissue behind pillars practically gone. Hemolytic streptococci disappeared from throat by 2nd week after treatment.

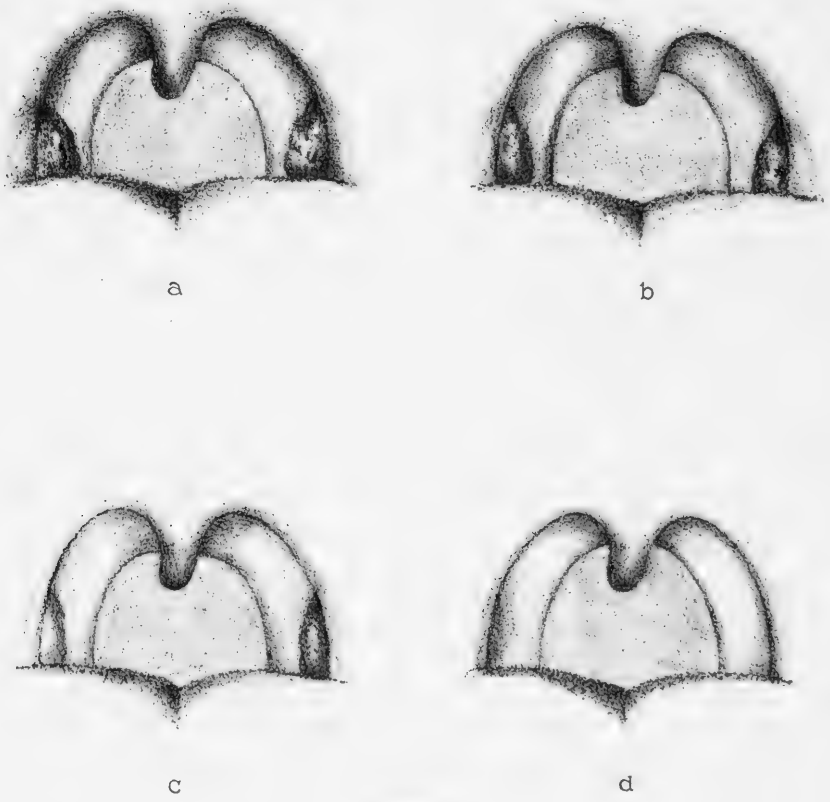


FIG. 1.

(Murphy, Witherbee, Craig, Hussey, and Sturm: Hypertrophied tonsils.)

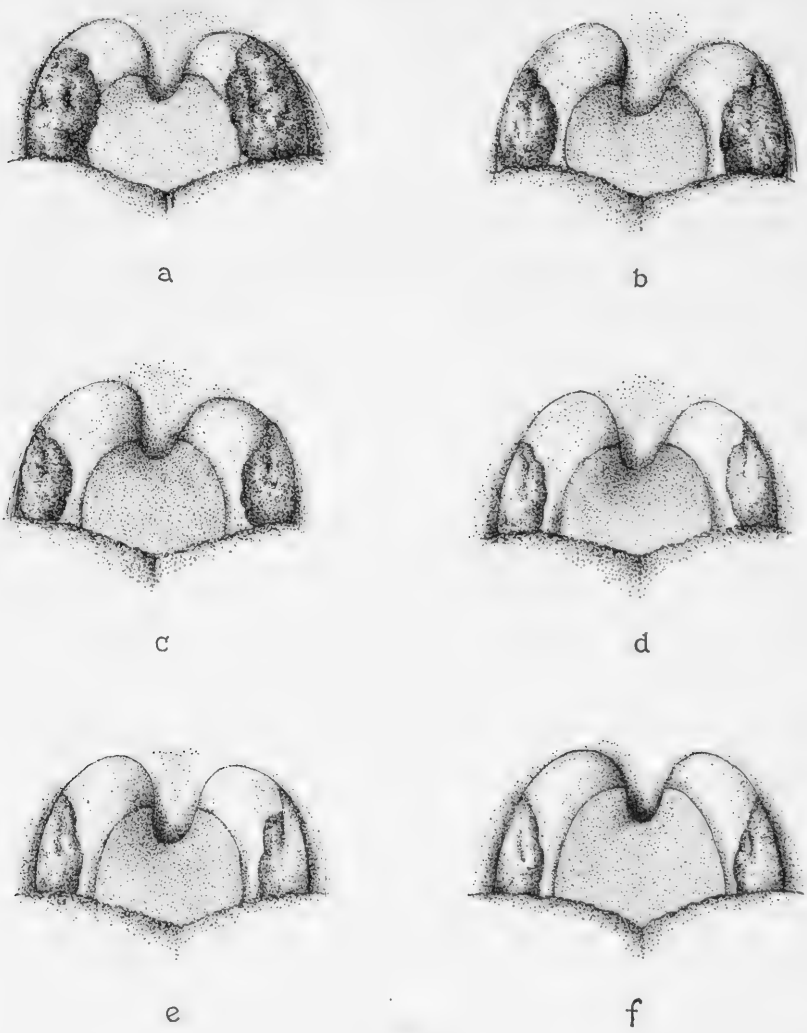


FIG. 2.

(Murphy, Witherbee, Craig, Hussey, and Sturm: Hypertrophied tonsils.)

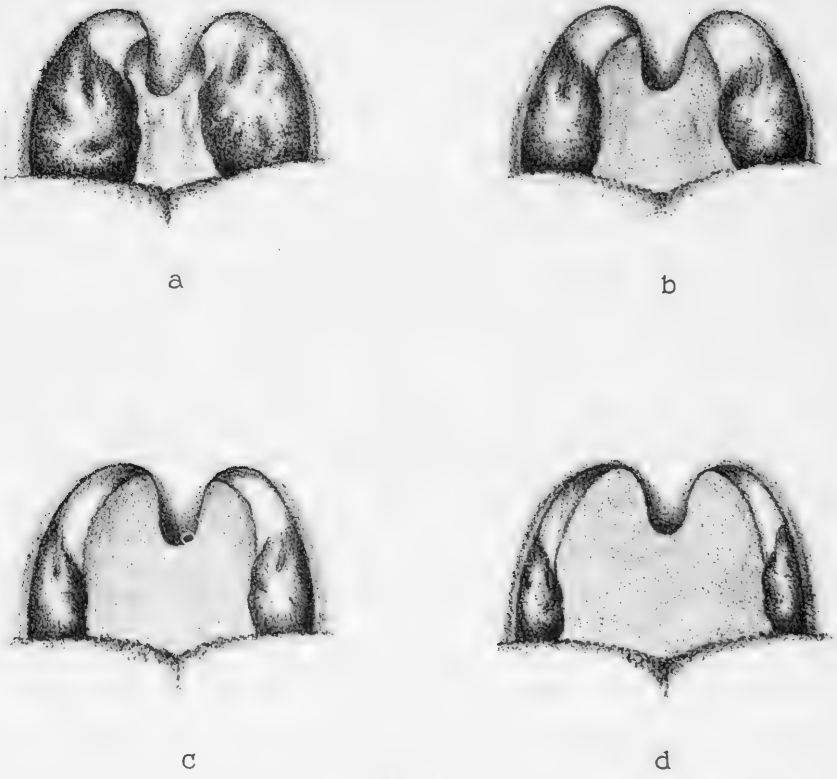


FIG. 3.

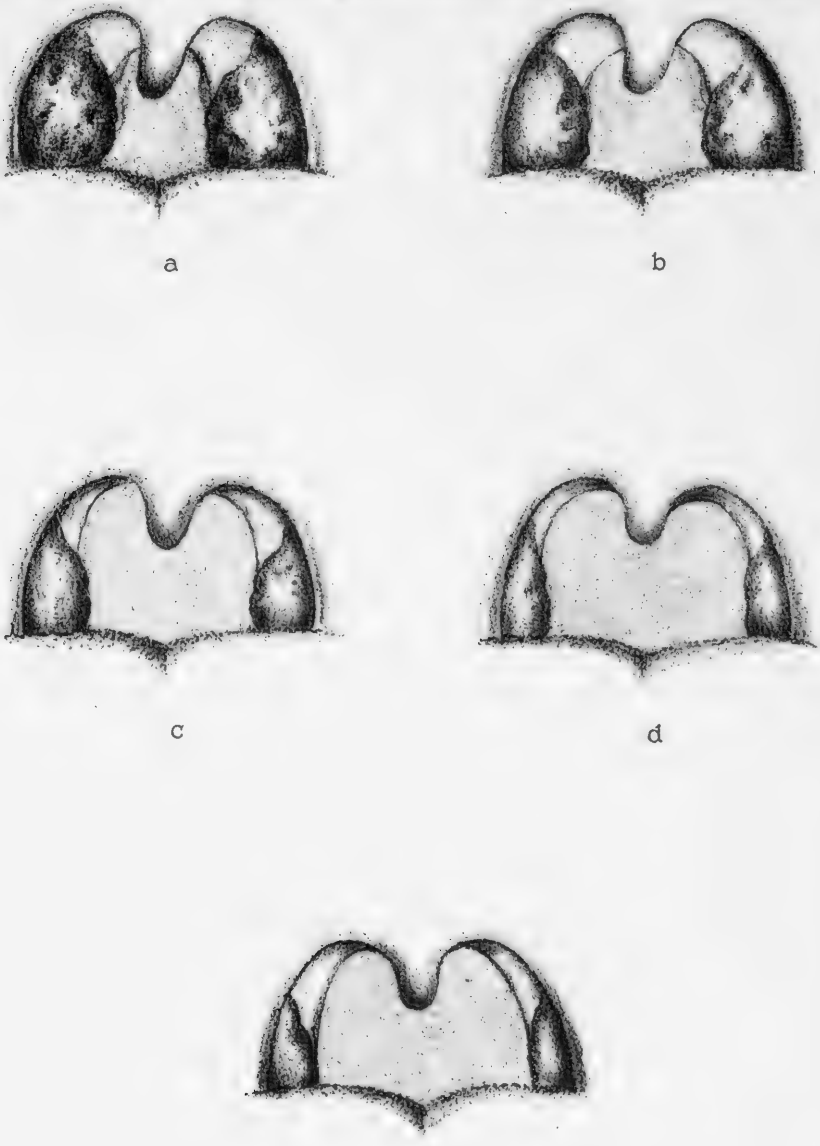


FIG. 4.

(Murphy, Witherbee, Craig, Hussey, and Sturm: Hypertrophied tonsils.)

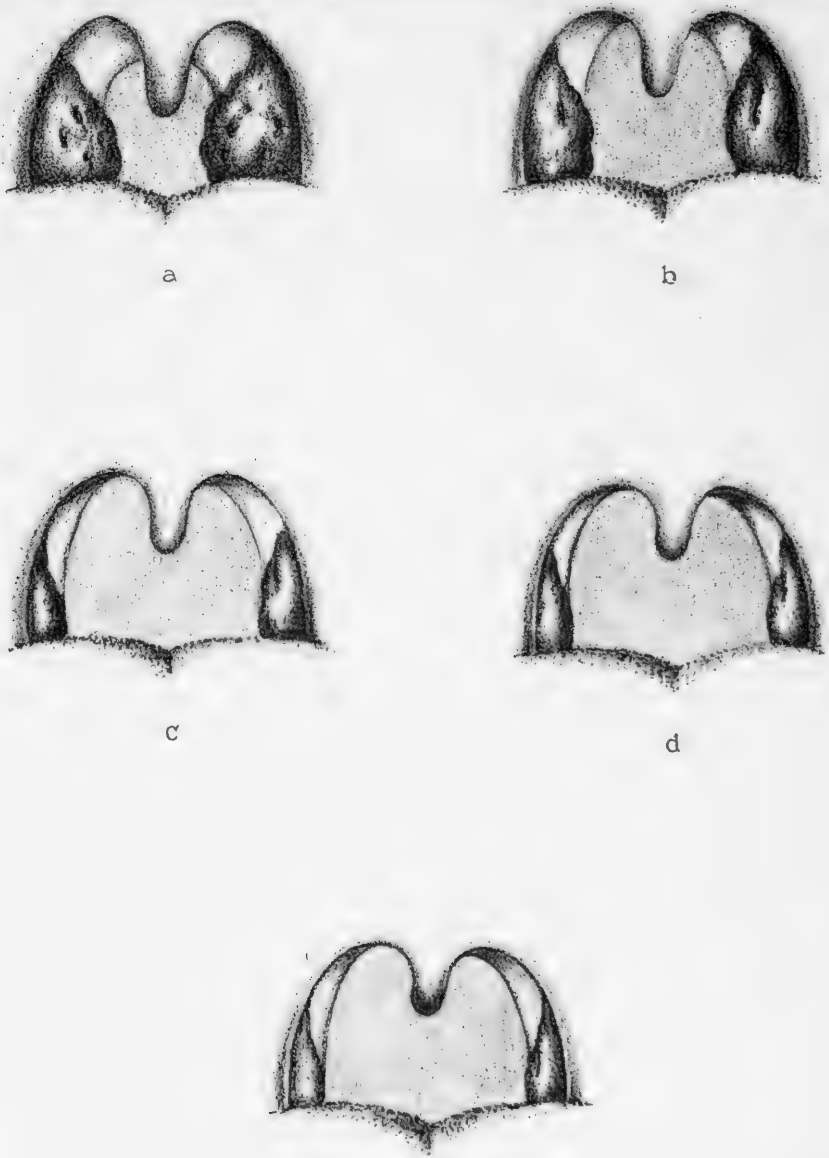
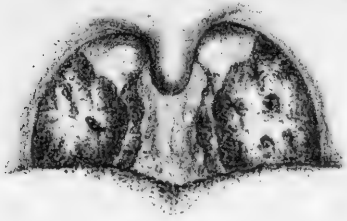


FIG. 5.

(Murphy, Witherbee, Craig, Hussey, and Sturm: Hypertrophied tonsils.)



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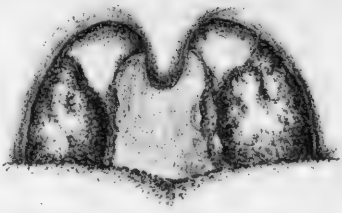

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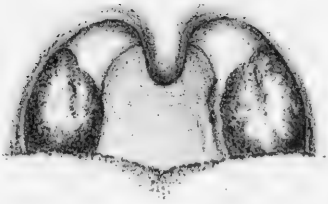

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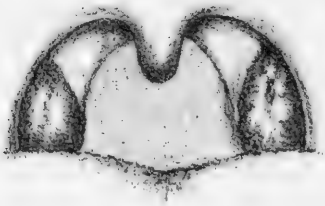

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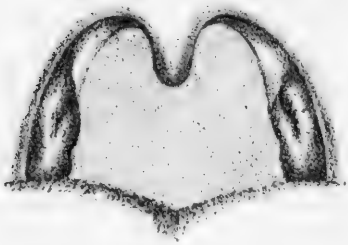

e

FIG. 6.

(Murphy, Witherbee, Craig, Hussey, and Sturm: Hypertrophied tonsils.)

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